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**The roles of selected
Mycobacterium tuberculosis genes
in DNA repair and pathogenesis**

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Abstract

Mycobacterium tuberculosis (Mtb), the bacterium which causes tuberculosis, is able to survive and replicate inside the hostile environment of the macrophage. Amongst other survival strategies, Mtb possesses mechanisms for repairing DNA damaged by exposure to reactive oxygen and nitrogen species produced by activated macrophages. Multiple DNA repair pathways exist and a number of genes of unknown function are induced under conditions of DNA damage.

In this study, the roles of previously uncharacterised genes predicted to be involved in the repair of damaged DNA, or induced by DNA damage, have been investigated by targeted mutation in Mtb. The genes under study are Rv0937c and Rv0938, homologous to Ku and ATP-dependent DNA ligase components of the non-homologous end joining system in eukaryotes, Rv2191, which shows a high level of homology to Cho, a recently discovered component of nucleotide excision repair in *E. coli*, and Rv3395c, which has been shown to be DNA damage inducible.

Mutant strains of Mtb were constructed with deletions in each of these genes, as well as a strain lacking both Rv0937c and Rv0938. These mutant strains were characterised in comparison with the wild type strain *in vitro*, following exposure to a variety of DNA damaging agents. The results revealed different patterns of heightened sensitivities when individual repair pathways were affected. Preliminary screens suggested a role in survival following exposure to oxidative damage for Rv2191 *in vitro* and further investigation confirmed this. This result

supports previous work implicating nucleotide excision repair for this type of damage in Mtb.

The ability of the strains to grow and survive in a mouse model of infection was assessed. The mutants deficient in components of the non-homologous end joining system were found to display attenuated growth in activated macrophages and possibly at late stages of infection in mice. This may suggest a potential role for non-homologous end joining during infection.

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Abbreviations

BER	Base excision repair
BCG	Bacille Calmette-Guerin
Bp	Base pair
BSA	Bovine serum albumin
cfu	Colony forming units
dH ₂ O	Distilled water
DC	Dendritic cell
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DNA PK	DNA-dependent protein kinase
DNA PK _{cs}	DNA-dependent protein kinase catalytic subunit
DOTS	Directly observed therapy, short course
Gm	Gentamycin
GSH	Glutathione
Gy	Greys
h	Hour
HR	Homologous recombination
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
Km	Kanamycin
Kb	Kilobase
MDR-TB	Multidrug resistant tuberculosis

MHC	Major histocompatibility complex
min	Minute
Msm	<i>Mycobacterium smegmatis</i>
Mtb	<i>Mycobacterium tuberculosis</i>
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NO	Nitric oxide
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RSNO	S-nitrosothiols
RT	Room temperature
s	Seconds
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride sodium citrate solution
TB	Tuberculosis
TBHP	Tertiary-butyl hydroperoxide
T _H	T helper
T _m	Melting point
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

1 Introduction

1.1 Tuberculosis

Tuberculosis (TB) is a leading cause of death from infectious diseases worldwide. Although the disease can be cured by a course of antibiotics and some form of protection is offered by the BCG vaccine, there were an estimated 8-9 million new cases and 2 million deaths in 2002 (Frieden *et al.*, 2003). It is thought that one third of the world's population is infected with the TB bacillus (WHO 2005).

Most reported cases of TB occur in less developed countries, with the highest incidence rate being in sub-Saharan Africa. This is currently reported at 290 per 100,000 population, with most cases occurring in individuals between 15 and 49 years old (Frieden *et al.*, 2003). As well as the need for improved vaccines, there is also the problem of the emergence and subsequent rise, of multi-drug resistant strains, especially in the former Soviet Union (WHO 2000). Multi-drug resistance, characterised by resistance to the drugs commonly used to treat TB, is hard to treat and is associated with high mortality (Kumar and Clarke 2005).

The most common symptoms of TB include coughing up blood, unexplained weight loss, night sweats and a general feeling of malaise. Although it is traditionally thought of as a lung disease, TB can affect multiple organs and body systems and can present with an alarming range of symptoms. TB can infect and grow in the heart, bones and reproductive system, to name but a few examples. Like other bacterial infections, TB can also spread to the brain in TB meningitis, a

particularly deadly form of the disease that primarily affects children. Therefore, common symptoms of TB may not be apparent, or symptoms may not be confined to a single organ, which can complicate and delay diagnosis.

1.2 *Mycobacterium tuberculosis*

The causative agent of tuberculosis is *Mycobacterium tuberculosis* (Mtb), which was originally described by Robert Koch in 1882 (Koch 1882). Mtb belongs to the mycobacteria family which also contains *M. leprae*, the cause of leprosy in humans, *M. bovis*, which affects a number of species including cows and badgers and *M. avium*, which affects birds, are also mycobacteria. The non-pathogenic and faster growing *M. smegmatis* (Msm) also belongs to the mycobacteria family.

Mtb is a Gram-positive, rod-shaped aerobic bacillus with a high GC content and a lipid-rich cell wall, thought to aid resistance to most antibiotics (Jarlier and Nikaido 1994). The cell wall accounts for around 10% of the total cell weight. Mtb is slow growing and undergoes periods of dormancy which are important in the pathogenesis of the disease.

The Mtb genome was sequenced in 1998 (Cole *et al.*, 1998) and re-annotated in 2002 (Camus *et al.*, 2002), enabling molecular insight into the disease and aiding investigation into potential causes of its pathogenicity via gene mutation studies and microarrays. The Mtb H37Rv genome is 4.4 million bp and has around 4000 genes, a large number of which are predicted to be involved in the generation and metabolism of lipids (Cole *et al.*, 1998). As was previously known, Mtb has natural resistance to selected antibiotics (Cole and Telenti 1995) and there are a

number of genes present in the genome that encode drug modifying enzymes including β -lactamases and aminoglycoside acetyl transferases (Cole *et al.*, 1998).

1.2.1 BCG Vaccination

At present, vaccination with BCG (Bacille Calmette-Guerin) is the only widely available preventative measure against TB, except for the use of TB prophylaxis in high-risk patients. The vaccine was developed in the 1920s by Messrs Calmette and Guerin by passaging a strain of *M. bovis* repeatedly over a period of 13 years until it was attenuated. The attenuation of BCG has been attributed to a series of deletions (Hsu *et al.*, 2003); a primary role has been attributed to the RD1 deletion which includes ESAT-6 and related proteins essential for virulence (Wards *et al.*, 2000). The BCG vaccine has not had a large impact on prevention of TB worldwide. Trials in the UK showed the vaccine to be around 75% effective (Fine 1995), but in some countries, the rate of protection is less than 10% (Fine 1995). BCG is most effective in protecting against serious childhood forms of TB, such as TB meningitis. However, up until recently, the vaccine was given as a matter of routine to older school children aged 13-15 in the UK. This practice has recently been stopped and instead BCG is given only to babies considered at risk (Hagan 2005). Previous infection with TB does not prevent re-infection (Mollenkopf *et al.*, 2004), so developing an effective vaccine is problematic.

1.2.2 Co-infection with HIV

A major factor in increased incidence of TB is co-infection with HIV and around 11% of new infections worldwide are reported to be co-infections (Corbett *et al.*, 2003). The risk of contracting TB is directly linked to CD4 T cell count, with a low count associated with contracting the disease. The majority of patients with AIDS actually die of TB and worldwide there are approximately 14 million people co-infected with HIV and TB (WHO 2005). As well as being more susceptible to TB, patients with HIV have a much higher chance of reactivating latent infection and disease progression can be much more severe in these cases. Despite the fact that TB infection is linked to low CD4 T cell counts, it has recently been found that in a cohort of gold miners in South Africa, the risk of contracting TB was doubled within the first year of infection with HIV, before the CD4 T cell count drops (Sonnenberg *et al.*, 2005). This may indicate a link between seroconversion and susceptibility to TB and suggests there may be a change in the immune system after infection with HIV which makes one more susceptible to TB (Sonnenberg *et al.*, 2005).

1.2.3 Treatment

TB is treated by a combination of drugs over an extended period (Fox *et al.*, 1999). Currently, treatment of TB is by DOTS, Directly Observed Therapy, Short course. Since its introduction by the WHO in 1995, it has proved very successful where implemented, and involves close supervision and monitoring of patients during their chemotherapy to make sure the entire course of drugs is completed, thus decreasing the likelihood of resistance developing. The treatment lasts 6-8

months and has a success rate of up to 95%, even in the poorest countries, with an average success rate of 82% (WHO 2004). However, success of treatment by DOTS depends on the availability of resources to implement the programme. In extreme cases of TB, infected sections of lung can be surgically removed in an effort to rid the patient of the disease, a technique commonly used to cure TB in the late 19th and early 20th centuries (Salyers and Whitt 2002).

TB is a difficult disease to treat owing to the long periods of dormancy associated with infection, and the difficulty of accessing bacteria deep within granulomas. In order to successfully cure the disease, long term treatment with a cocktail of antibiotics is necessary. Using two or more antibiotics decreases the chance of resistance developing and the current regimen includes 2 phases, the first intensive phase is intended to kill actively growing bacteria and to limit the infectiousness of the patient. The second phase is designed to target any remaining bacteria and to reduce the relapse rate to an acceptable level. Isoniazid and rifampicin are used in the initial phase, usually with pyrazinamide and ethambutol for 2 months (Frieden *et al.*, 2003), followed by 4 months of just isoniazid and rifampicin. The duration of treatment varies depending on the drugs given and severity of the infection. The treatment also depends on the HIV status of the individual as some antiretroviral drugs used to treat HIV can adversely affect the bactericidal action of rifampicin.

1.2.4 Multidrug Resistance

Multidrug resistant TB (MDR-TB) is a worrying phenomenon, exacerbated by non-compliance with drug regimes and other socio-economic factors. MDR-TB is

defined as a strain of TB resistant to isoniazid and rifampicin, two of the most powerful and commonly used anti-TB drugs, (WHO 2004) and is thought to be exacerbated by error prone repair of DNA damage induced by the host immune system (Boshoff *et al.*, 2003) or by the drugs themselves. Drug resistance in Mtb is chromosomally encoded (Ramaswamy and Musser 1998). In other pathogenic intracellular bacteria, induction of the SOS response following DNA damage incurred during infection has been shown to increase the mutation rate (Schlosser-Silverman *et al.*, 2000). In Mtb, it has been demonstrated that DNA damage increases mutation rates by a mechanism independent of the induction of known error-prone polymerases but dependent on the *dnaE2* polymerase (Boshoff *et al.*, 2003). The expression of this gene was upregulated in wild-type Mtb during infection *in vivo* and the Mtb *dnaE2* knockout mutant showed attenuation as well as a reduced frequency of drug resistance in a mouse model (Boshoff *et al.*, 2003), which suggests a role in mutation following exposure to DNA damaging free radicals within the host.

Strains of Mtb have been isolated that are resistant to all major anti-TB drugs. MDR-TB is very difficult to treat and carries a high risk of fatality. It has recently been found that around 10% of all new cases in some countries in the former Soviet Union are multi drug resistant (WHO 2000). Despite the relatively low frequency of resistance to single drugs seen *in vitro*, MDR-TB *in vivo* is becoming increasingly common with between 1-3% of clinical isolates resistant to more than one anti-TB drug (Espinal *et al.*, 2000). Multidrug resistant TB is treated by using at least 3 or 4 different drugs taken orally, as well as 1 injected drug, to which the bacteria are thought to be fully susceptible, for the initial 3 month period (WHO

2004). The treatment can be very lengthy and often requires an increased number of drugs to be taken by the patient, which can have an adverse effect on compliance. The drugs must be taken by the patient for 12-18 months after culture conversion to negative for TB (WHO 2004).

1.3 Pathology and disease progression

TB is spread by aerosol and disease can occur either by primary inhalation or by reactivation of an old lesion. The spread by aerosol is highly effective as it ensures that the infectious droplets bypass the immune cells of the upper respiratory tract and are delivered straight into the lungs. The droplets can also remain in the air for some time after they are coughed up by an infectious individual due to their small size (Salyers and Whitt 2002). Transmission generally occurs after prolonged contact between a susceptible individual and an individual suffering from active TB.

Once the bacilli have been inhaled by a susceptible individual, the bacteria reach the lungs where they enter the macrophages and multiply. At this point, if the bacteria can overcome the initial host defences, successful infection can result in an asymptomatic primary tuberculosis within the lungs. Primary tuberculosis is defined as developing within 1 or 2 years of exposure (van Crevel *et al.*, 2002).

Mtb can survive for long periods in unactivated macrophages so whether the susceptible individual develops the disease may depend on their ability to form a rapid immune response. Initially, neutrophil granulocytes reach the bacteria in the

lungs and engulf them. Alveolar macrophages, which phagocytose the bacteria, are recruited to the site, and present the peptide fragments from engulfed bacteria to T helper cells via MHC class II, thus activating the adaptive immune response. As well as stimulating the production of antibody, macrophages trigger the production of cytokines including IFN- γ , which in turn activates phagocytic cells. Activated phagocytes release a respiratory burst, which impacts on intracellular killing.

The initiation of this cell mediated response is indicated by a positive tuberculin skin test. A delayed type hypersensitivity reaction at the site of the lesion then follows and the infection is walled off inside a granulomatous lesion, encompassing a central area of caseous necrosis. However, a positive skin test is not an indication of infectious TB and only around 1% of skin test positive individuals actually develop clinical TB. More than 90% of infected individuals remain free of clinical disease for the duration of their lifetime. This indicates that although the human immune system cannot actually eradicate the bacterial infection, in the majority of cases it can control it relatively effectively, as long as the immune system remains unchallenged by immunodeficiency.

As mentioned previously, bacteria multiplying in the macrophage can cause the formation of lesions and macrophage differentiation leads to the formation of granulomas. Granulomas are made up of macrophages, T cells, B cells, fibroblasts and epitheloid cells which merge to form giant multinucleate cells (Flynn and Chan 2001). A zone of necrosis forms at the centre of the granuloma and collagen fibres may also be deposited causing fibrosis at the periphery. The function of the

granuloma is to isolate the bacteria, and restrict growth of the phagocytosed bacteria by confining the infection to suboptimal conditions, thought to include low aeration and nutrient depletion. In some cases the bacteria stop growing altogether and the lesion calcifies. This solidified area is visible on chest X-rays and is a good diagnostic indication of TB infection. The calcified lesion remains so, unless the host undergoes a period of immune suppression which can cause reactivation. This is known as 'post primary tuberculosis', the symptoms of which include those commonly associated with the effects of cytokine activation such as night sweats, fever and weight loss. The persistent cough, often accompanied by blood stained sputum is due to lung cavitation and associated damage. When the lesion liquefies, bacteria are coughed up, spreading TB to other susceptible individuals and at the same time bacteria can spread to other organs via the bloodstream, ultimately leading to death.

The outcome of exposure and subsequent infection with *Mtb* is known to be highly variable and dependent upon a number of factors (van Crevel *et al.*, 2002). Severity of the disease appears to depend on the balance between the host immune system and the microbe's ability to evade, or withstand the host immune response. The spectrum of TB infection can range from a relatively benign TB pleuritis confined to the lungs, to a raging disseminated and usually fatal miliary TB (van Crevel *et al.*, 2002).

1.3.1 Latency and persistence

TB is an unusual disease in that following infection, the bacteria can lie dormant in macrophages for decades until an event to trigger their reactivation. This is

most commonly due to immune suppression, which can be caused by a variety of factors including HIV, old age or chemotherapy.

The location of the latent bacteria is unknown. Occasionally in a case of reactivated TB, the patient can have the infection in an organ other than the place of the original infection. For example, an infection that was initially confined to the lung can reactivate in the kidney. This raises the possibility that latent bacteria are not only in the lung and also that they are able to move around the body prior to latency via the lymphatic system (Frieden *et al.*, 2003). Dormant bacteria are thought to be subjected to low level attack from the human immune system in the form of the damaging defence mechanism produced by macrophages.

The exact mechanism by which the bacteria can survive for so long is not fully understood and it has been postulated that they take on a spore like state, where metabolism virtually shuts down. One of the sigma factors identified in the Mtb genome (Cole *et al.*, 1998), *sigF*, is homologous to a gene in *B. subtilis* which controls responses to environmental stress (DeMaio *et al.*, 1996). In Mtb, *sigF* is expressed during growth in stationary phase and has been shown to play a role in persistence *in vivo* (Chen *et al.*, 2000). Other genes have also been implicated in the survival of Mtb during persistence. These include *acr*, which encodes α -crystallin and is implicated in survival under anaerobic conditions (Wayne 1994), and the gene coding for isocitrate lyase (McKinney *et al.*, 2000), which catalyses the reaction from isocitrate to glyoxylate in the glyoxylate cycle, allowing the bacteria to bypass the tricarboxylic acid (TCA) cycle.

Unfortunately, there are no entirely accurate animal models for latent TB, which raises problems for our understanding of the exact mechanisms involved. Although mice are generally used, they develop persistent infection 2-4 weeks after the initial inoculation with Mtb and despite high levels of bacteria in the lungs, the mice remain reasonably healthy and can survive for around a year (Flynn and Chan 2001). Mice do not develop granulomas, where the bacteria are contained and maintained at a low level, although they can be treated with chemotherapy to reduce the numbers of bacteria and artificially create an animal model more similar to humans in terms of pathology. Upon withdrawal of the antibiotics, the infection reactivates spontaneously or as a consequence of immunosuppression (McCune *et al.*, 1966). This is known as the Cornell model and is used as a model for latent infection but is regarded as being somewhat unreliable. Aside from mice, guinea pigs, rabbits and non-human primates are occasionally used as animal models for TB. The rapid disease progression in guinea pigs makes developing a model for latent TB in this species difficult, whilst the larger sizes of the other animals limits the practicability of their widespread use.

1.3.2 Survival under oxygen and nutrient limiting conditions

Although Mtb is an aerobic organism, many of the conditions encountered by the bacterium during the later stages of infection are anaerobic with low pH and low nutrient concentrations. It has been shown that the bacteria are able to survive under these conditions *in vitro* by adapting to a state of non-replicating persistence (Wayne 2001). When bacteria enter these conditions gradually, their viability is unaffected and metabolism shifts into a dormant state (Wayne 1976). Upon a

change to more favourable conditions, the bacteria can regain their viability. However, if the change from plentiful oxygen to low oxygen occurs suddenly, actively replicating bacteria are killed instantly (Wayne and Diaz 1967).

The pattern of dormancy and the ability to survive in oxygen limiting conditions makes it hard to treat the bacterial infection. Most currently available antibiotics target rapidly dividing and growing cells, and so their effectiveness on dormant bacteria is limited. Dormant bacteria are also thought to be difficult to treat due to their location, often in granulomas, *in vivo*. It is not known how effectively antibiotics penetrate these structures to access the bacteria.

As well as being able to survive in conditions of oxygen depletion, mycobacteria are able to survive under conditions of nutrient limitation. In a similar manner to the response to reduced levels of oxygen, mycobacteria can reduce their rate of respiration to a minimum level during nutrient depletion, and upon an increase in nutrient availability recover their metabolic state (Loebel 1933). This reactivation has been shown to be possible after surviving starvation conditions for up to 2 years. It has been hypothesised that *in vivo*, Mtb changes its metabolism to avoid nutrient starvation and utilises lipids as a carbon source, a process for which it possesses a number of suitable enzymes (Cole *et al.*, 1998). The environment inside granulomas is lipid rich and, therefore, can support bacterial survival under these conditions.

It has also been demonstrated that the stringent response plays a role in long term survival of Mtb. The stringent response involves hyperphosphorylated guanine as

a signalling molecule which can control gene expression during nutrient depletion. A mutant unable to produce this protein showed a severe growth defect and was unable to persist in anaerobic conditions (Primm *et al.*, 2000). Furthermore, this mutant strain was defective in persistence in mice (Dahl *et al.*, 2003).

1.4 The Human Immune Response

The immune system in humans is made up of 2 components, the innate and adaptive immune systems (Schluger and Rom 1998).

1.4.1 Innate immunity

The innate response consists of physical and chemical barriers, as well as phagocytic and natural killer cells, blood proteins including members of the complement system, and cytokines. The innate immune response provides the early lines of defence against microbial infection and recruits macrophages and inflammatory cells to the site of infection (Rosenberger and Finlay 2003), as well as presenting antigen bound to the major histocompatibility complex for recognition by T cells. T cells in turn stimulate antibody production via B cells, and together form the adaptive immune response. The main purpose of innate immunity is to control the infection whilst the adaptive immune response is initiated. The importance of the innate immune response is highlighted by the fact that its absence or compromise, as seen in AIDS, is associated with increased reactivation of latent TB infection, and an increased likelihood of contracting TB or other less common mycobacterial infections.

The macrophage has a crucial role in Mtb infection and has a central position in pathogenesis and disease progression. It is a vital component of the immune system in the battle against the Mtb bacillus and the outcome of infection, i.e. whether the individual develops TB infection, is largely attributed to the macrophage. During infection, the macrophage carries out a number of functions including engulfment of the bacilli, presentation of peptide fragments to T cells with subsequent activation of adaptive immunity, and cytotoxic killing of the engulfed bacteria, which is stimulated in part by the release of cytokines from T helper cells. Defects in any of the macrophage functions have serious consequences for the host.

1.4.2 Adaptive immunity

Adaptive immunity is induced by the presence of antigen and is a specific and adaptive response to the infection, occurring approximately 2-4 weeks post infection in the mouse model. Adaptive immunity involves lymphocytes and induces humoral and cellular immunity, which is thought to be the most important defence against Mtb. Cell mediated immunity is controlled by T cells, which recognise the microbial antigens presented on the surface of infected cells. This in turn leads to the production of cytokines to activate phagocytes. CD4⁺ and CD8⁺ T cells both play an important role in fighting infection with Mtb (Boom 1996). CD4⁺ T cells are stimulated to differentiate into helper T cells (T_H1) cells by cytokines including IL-12 and IL-18 (Flynn and Chan 2001), which are themselves induced via Toll-like receptors (Means *et al.*, 1999). The T_H1 cells are potent producers of IFN- γ , which activates macrophages to kill Mtb. Interestingly, T_H2 cytokines are less common in Mtb infection (Lin *et al.*, 1996). Mtb can also

stimulate MHC-class-I-restricted CD8⁺ T cells (Kaufmann 2001) which also play a role in the immune response against infection with Mtb (Kaufmann 2000) and can produce IFN- γ , as well as killing target cells (Flynn and Chan 2001).

Dendritic cells have also been found to play an important role in the control of Mtb infection (Tascon *et al.*, 2000) and express high levels of MHC Class II molecules which aid recognition of the pathogen via T cells. Dendritic cells, which are highly motile, are thought to be the principle antigen presenting cells (Rosenberger and Finlay 2003) and are able to activate naïve T cells.

It has been suggested that Mtb antigen presentation is potentially down-regulated during infection, to prevent the activation of intracellular killing mechanisms (Noss *et al.*, 2000). However, the opposite has been suggested for *in vitro* antigen presentation by dendritic cells (Henderson *et al.*, 1997) but details of this process *in vivo* are as yet unconfirmed.

1.4.3 Recognition and phagocytosis of Mtb

Microbial recognition by the innate immune system is achieved by macrophages via a number of different receptor types. Consequently, Mtb, an intracellular pathogen, has evolved numerous mechanisms to recognise and exploit these receptors to gain entry into macrophages. The receptors can either bind to the bacteria directly via components located on the bacterial cell surface, or indirectly where the bacteria are coated in opsonins such as complement as a result of adaptive immunity.

Toll-like receptors (TLRs), which are a family of transmembrane proteins, play an important role in recognition of Mtb (Means *et al.*, 1999). TLRs are able to recognise Mtb Lipoarabinomannan (LAM) and bind to various domains which trigger activation of transcription factors to produce cytokines involved in the inflammatory response (Oddo *et al.*, 1998). TLRs bind with a cofactor, MyD88, which is thought to play a part in macrophage activation following Mtb infection.

Pattern Recognition Receptors (PRR), are a set of germline encoded proteins which can recognise pathogen associated microbial patterns (PAMPs) (Medzhitov and Janeway 1997). At the first signs of infection, the PRRs recognise PAMPs such as bacterial lipopolysaccharide or lipoteichoic acid (Medzhitov and Janeway 1997). Receptors on the host phagocytes bind to the bacterial PAMPs, which can be used by the pathogen as a means of gaining entry into the host cells.

Following recognition of Mtb, uptake for phagocytosis occurs via a range of receptors including complement receptors, mannose receptors and surfactant protein receptors on the host macrophage. It is thought that this initial binding stage may in some way affect the ensuing infection and subsequent survival of the bacteria (Brown and Gordon 2002). For example, Mtb exploit the complement receptors to gain entry into macrophages in a range of different ways and use them to manipulate the host immune response. Additionally, mannose receptors expressed on mature macrophages can affect the production of the respiratory burst and interestingly, phagocytosis through the mannose receptor inhibits the respiratory burst (Astarie-Dequeker *et al.*, 1999). However, entry through the

easily exploitable mannose receptor is arrested by the presence of IFN- γ (Schreiber *et al.*, 1993), which inhibits mannose receptor expression.

1.4.4 Outcome of infection

In order to be able to generate a successful infection, it is imperative that the bacteria survive the host immune response. They can do this in one of two ways, or use a combination of both.

Firstly, the bacilli can evade the defence mechanisms. For example, entering the macrophage through specific receptors will down-regulate the respiratory burst and consequent intracellular killing. Furthermore, it has been demonstrated that pathogenic strains of Mtb can inhibit phago-lysosome fusion, thus reducing their exposure to acidic pH and proteolytic enzymes (Armstrong and Hart 1975). Secondly, the bacteria can attempt to repair any damage sustained as a result of exposure to the host immune response.

The exact order of events following infection with Mtb and whether repair or evasion plays a more important role in bacterial survival *in vivo* is controversial. However, during Mtb infection, antigen presentation by infected cells can be altered so that the CD4⁺ T cells do not detect the infection, and therefore macrophages are not activated by cytokines such as IFN- γ and TNF- α to release RNI and ROI (Flynn and Chan 2001). For macrophages to become activated, CD4⁺ T cells must recognise infected macrophages via MHC class II presentation of mycobacterial antigens and it has been demonstrated in murine macrophages.

that infection with Mtb can inhibit this antigen presentation, potentially through the role of a recently discovered 19kDa lipoprotein (Noss *et al.*, 2000).

1.4.5 Growth factors and cytokines

Successful internalisation and intracellular killing of the bacteria are followed by antigen presentation of the Mtb peptide fragments and the release of growth factors and cytokines. These factors released by macrophages after phagocytosis recruit and activate other cells involved in the immune response and inflammatory process, with the outcome of further increasing macrophage activation. This in turn leads to further intracellular killing and thus modulation of the immune response. Growth factors are important in increasing macrophage numbers during Mtb infection and GM-CSF is important in monocytoysis. Chemokines, produced predominantly by macrophages, are crucial in the recruitment of inflammatory cells to the site of infection.

A plethora of cytokines are released by macrophages during Mtb infection and these include both pro- and anti-inflammatory cytokines. Additionally, IL-6 has both pro- and anti-inflammatory properties, and its deficiency is associated with fatal Mtb infection (Ladel *et al.*, 1997).

The pro-inflammatory cytokines include the interleukins IL-1 β , IL-6, IL-12, IL-15, IL-18, IFN- γ and TNF- α . IFN- γ , produced by T helper 1 cells, activates macrophages and its importance in Mtb infection is demonstrated by the fact that humans and mice unable to produce it are more susceptible to Mtb infection (Jouanguy *et al.*, 1999). Furthermore, mice deficient in IFN- γ production are

unable to activate macrophages and consequently, are susceptible to fatal TB infections (Flynn *et al.*, 1993). Prior to the initiation of adaptive immunity, it is thought that Natural Killer cells produce IFN- γ (van Crevel *et al.*, 2002). TNF- α is essential for granuloma formation (Senaldi *et al.*, 1996) and additionally induces macrophage activation. However, this comes at a price and it is TNF- α which is a main contributor to the pathology associated with the disease. A neutralising TNF- α antibody administered to mice decreases pathology but increases bacterial load (Mohan *et al.*, 2001).

Mtb induces a range of anti-inflammatory cytokines, which include IL-4, IL-10 and TGF- β . These cytokines antagonise the pro-inflammatory cytokines and can be looked upon as a fine tuning mechanism for regulation of the immune response. IL-12 induces IFN- γ production and thus is important in increasing intracellular killing. Conversely, IL-10 down regulates the production of IFN- γ and overproduction of IL-10 is associated with a deficient immune response to Mtb and therefore increased susceptibility to disease (Boussiotis *et al.*, 2000).

1.5 Killing mechanisms

Intracellular killing is initiated by the activation of macrophages triggered by cytokines. The macrophages are activated by IFN- γ and TNF- α produced by T-helper cells, which were themselves stimulated to produce cytokines by macrophages presenting MHC bound Mtb peptide fragments.

Infected macrophages activated by IFN- γ and TNF- α , release reactive oxygen and nitrogen intermediates, which can cause a range of damage to bacterial lipids, proteins and DNA. Damage to DNA is thought to be of particular importance, and this can take the form of oxidation, deamination or alkylation. Such damage can result in single or double strand breaks in the DNA if left un-repaired. It has been demonstrated that the products of reactive oxygen intermediates (ROIs), and reactive nitrogen intermediates (RNIs), superoxide and nitric oxide respectively, can react together to form peroxynitrite, a strong oxidant with DNA damaging properties (Shiloh and Nathan 2000). As well as exposure to ROIs and RNIs in activated macrophages, bacteria are subjected to nutrient deprivation, hydrolytic enzymes and an acidic environment, with the added challenge of oxygen limitation in the granuloma.

Glutathione, an antioxidant present in most cells, protects host cells from the effects of ROIs and RNIs (Seres *et al.*, 2000) and is synthesised and released alongside them. Glutathione has a number of additional roles, including regulation of antigen processing and regulation of Th1 and Th2 cell mediated immunity (Peterson *et al.*, 1998). Recently, it has been proposed that glutathione plays a direct role in killing mycobacteria due to its reaction with NO to produce S-nitrosoglutathione (GSNO) (Venketaraman *et al.*, 2003). GSNO acts as a NO donor and contributes to increasing the activity of NO. Release of NO from GSNO has a bactericidal effect on mycobacteria, as would be expected. Its effects are similar to those demonstrated following exposure to RNI (Chan *et al.*, 1992). Further work has confirmed these findings in Mtb and demonstrated that control

of intracellular growth of *Mtb in vitro* is dependent upon glutathione (Venketaraman *et al.*, 2003).

1.6 Reactive Nitrogen and Oxygen Intermediates

Reactive nitrogen and oxygen intermediates oxidatively damage DNA, resulting in a range of DNA alterations including abasic sites and strand breaks.

1.6.1 RNIs

The production of nitric oxide (NO) is one of the macrophages' most important defence mechanisms. NO can cause single and double strand breaks in the bacterial DNA. In addition, nitrosative stress leads to deamination of DNA bases *in vitro* (Wink *et al.*, 1991), for example C→U, which is mutagenic and damaging to the bacterial DNA. Peroxynitrite, damages bacterial proteins via a number of different mechanisms and is additionally associated with membrane damage (Fang 1997).

NO is produced in mammalian cells by enzymatic oxidation of L-arginine and is involved in a diverse range of physiological processes, amongst them, antimicrobial activity. The production of NO in macrophages is catalysed by the inducible nitric oxide synthase, or iNOS, which can bind calmodulin without depending on elevated levels of Ca²⁺ ions. iNOS is expressed in granulomas in the lungs of infected mice and is essential for the production of RNI in these tissues (Flynn *et al.*, 1998). A link has been shown between the level of virulence of a strain and its resistance to the toxic effects of RNI (O'Brien *et al.*, 1994). It has

also been shown that cytokine induced inhibition of Mtb growth in macrophages is linked to the production of RNI (Chan *et al.*, 1992). Although the roles of RNI in mycobacterial killing in a mouse model during the initial stages of infection have been clearly demonstrated in a number of studies (Dalton *et al.*, 1993; Chan *et al.*, 1995), the role during persistent infection is less well defined. RNI production is necessary to prevent reactivation of persistent infection in mice (Flynn *et al.*, 1998) and it is proposed that this is due to RNI having a bacteriostatic effect on Mtb and preventing it from proliferating during infection in mice (Firmani and Riley 2002).

Interestingly, it has been proposed recently that low concentrations of nitric oxide actually enhance mycobacterial growth *in vitro* (Brugmann and Firmani 2005) and may actually encourage persistence. This may be an adaptive response by the bacteria to enable survival in otherwise harsh conditions.

1.6.1.1 Mechanisms of nitrosative damage to DNA

Nitrosative damage occurs either directly, or via the reaction of RNI with ROI to form peroxynitrite. The damaging effects of nitric oxide are varied, and the extent of damage sustained depends on its rate of production and diffusion, the concentration of reactants such as superoxide, and the levels of neutralising enzymes and antioxidants (Marletta 1988).

As well as the NO radical itself (NO \cdot), other inducers of nitrosative damage include nitrogen dioxide (N $_2$ O), dinitrogen trioxide (N $_2$ O $_3$), and dinitrogen tetroxide (N $_2$ O $_4$). Additionally, NO $_2^+$, a powerful oxidant, is formed by the auto-

oxidation of NO^\cdot (Eiserich *et al.*, 1996). S-nitrosothiols such as S-nitrosogluthathione are also damaging to bacterial DNA and are formed from NO^\cdot and reduced thiols in the presence of an electron acceptor (Gow *et al.*, 1997). The two main reactions of NO in biological systems are auto-oxidation to form nitrous anhydride, N_2O_2 and the reaction with superoxide to form peroxynitrite (Tamir *et al.*, 1996). Peroxynitrite, (OONO^\cdot), oxidises and nitrates DNA, and through its attack on the sugar phosphate backbone, can cause single strand breaks (Burney *et al.*, 1999). Peroxynitrite reacts most commonly with guanine and causes G:C to A:T transitions. Nitrous anhydride can cause deamination and subsequent crosslinks in DNA via nitrosation of primary amines in DNA bases (Burney *et al.*, 1999).

1.6.2 ROIs

Superoxide production is stimulated by cytokines released by the inflammatory response via phox, the phagocyte NADPH oxidase complex. Superoxide can then be converted into hydrogen peroxide by the enzyme superoxide dismutase (SOD). There has been some debate over the importance of the role of ROI during *Mtb* macrophage infection and it has been shown that ROI deficient cell lines show similar mycobactericidal killing activity to those with the ability to generate ROI (Chan *et al.*, 1992). However, more recently it has been found that mutant mice which cannot generate an oxidative burst, show decreased survival compared to wild type mice following infection with another intracellular pathogen, *Listeria monocytogenes* (Shiloh *et al.*, 1999). In support of this, it has been demonstrated that the reactive oxygen burst is a cause of severe damage to intracellular bacteria (Schlosser-Silverman *et al.*, 2000) as *S. enterica* serovar typhimurium (*S.*

typhimurium) *recA* and *recBC* mutants are attenuated in mice. These mutated genes are components of homologous recombination, a DNA repair pathway. The mutant strains display decreased survival in macrophages, implying that the macrophage environment is damaging to the DNA (Buchmeier *et al.*, 1993). The bacterial DNA damage induced by the macrophage resembles the pattern of damage caused by ROI, consisting of lesions such as single point mutations and single strand breaks (Schlosser-Silverman *et al.*, 2000).

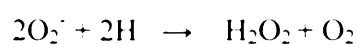
A comparison between the susceptibility of *E. coli*, which is sensitive to the intracellular macrophage environment, and *S. typhimurium*, found that *E. coli* inhibits its gene expression within macrophages and incurs significant levels of DNA damage. In contrast, *S. typhimurium*, which is an intracellular pathogen and like Mtb can survive and replicate within macrophages, was much less susceptible to DNA damage within macrophages (Schlosser-Silverman *et al.*, 2000). *S. typhimurium* can reduce its exposure to DNA damaging agents through a type III protein secretion system, encoded by *Salmonella* pathogenicity island 2 (SPI2) (Vazquez-Torres *et al.*, 2000). SPI2 changes the target of phox away from the vacuole containing the bacteria and SPI2 mutants show decreased survival in macrophages and reduced virulence in mice (Vazquez-Torres *et al.*, 2000). It has also been demonstrated that *S. typhimurium* can change the targeting of iNOS from the vacuole to elsewhere (Chakravorty *et al.*, 2002). As well as being able to divert the actions of phox and iNOS, *S. typhimurium* has evolved enzymes that can detoxify RNI and ROI (Webb *et al.*, 2001)

Microarray data following Mtb exposure to oxidative stress conditions showed a marked induction of a number of genes associated with DNA repair and survival under stress, such as *recG* and *sigB* (Schnappinger *et al.*, 2003). This supports the fact that ROI (and RNI) may have a role in controlling the infection, in association with other immune defence mechanisms, and that it is likely that like other intracellular pathogens such as *S. typhimurium*, Mtb is able to both evade and survive the immune response as necessary.

1.6.2.1 Mechanisms of oxidative damage to DNA

As described previously, DNA is a target of reactive oxygen species and the damage incurred can be lethal for the bacteria. Reactive oxygen species can cause a wide range of damage to bacterial DNA, most notably the modification of guanine residues.

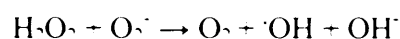
Free oxygen is relatively unreactive with DNA but when the oxidation state and, therefore, electron configuration is changed, the resulting oxygen ions can attack and damage the DNA. Partial reduction of oxygen results as the unstable oxygen reacts with other compounds by accepting electrons to form the superoxide radical O_2^- . Superoxide radicals are formed in phagocytic cells and neutrophils which use NADPH oxidase to reduce oxygen to superoxide radicals, thereby generating a killing response inside the macrophage. The superoxide radical can also dismutate to form hydrogen peroxide:



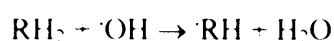
The hydrogen peroxide can further react via the Fenton reaction (Fenton 1894) using Fe^{2+} ions, to form the hydroxyl radical:



Alternatively, hydroxyl radicals can be formed via the Haber-Weiss reaction where the superoxide radical reacts with hydrogen peroxide directly.



Hydroxyl radicals ($\cdot\text{OH}$) are very damaging to DNA and can cause a range of modifications, including the release of free bases (ie base loss), generating simple abasic (AP) sites and strand breaks with a terminal sugar residue fragment. 8-oxoG is an example of oxidative damage to dG, which occurs through the addition of a hydroxyl radical to the C-8 position of guanine (Jovanovic and Simic 1989). If unrepaired, this causes mispairing, leading to an increase in G to T transition mutations. Hydroxyl radicals can also attack thymine at the C5-C6 double bond, forming a 6-hydroxythymine radical intermediate which reacts with oxygen to form thymine glycol (Demple and Linn 1982), which in turn, acts to block DNA replication (Clark and Beardsley 1987). One very damaging effect of hydroxyl radicals is their ability to initiate chain reactions causing damage some distance away from the initial event (Saran and Bors 1990). Chain reactions start from the initial event of the hydroxyl radical reacting with residues of organic macromolecules:



As mentioned previously, superoxide radicals can also react with nitric oxide in a number of different ways to form the peroxynitrite radical, (OONO[•]), a very strong oxidant which is severely damaging to bacterial DNA.

1.7 Intracellular survival pathways

If the bacteria are to survive inside macrophages, it is of the utmost importance that they either manipulate the conditions to make them more favourable or can adapt in some way to withstand the conditions, such as by repairing any damage sustained.

1.7.1 Inhibition of phagosome maturation

The ability of Mtb to survive in macrophages is thought to be due in part to its ability to arrest the maturation of phagosomes (Armstrong and Hart 1975) and so to reside in compartments which are secluded from the late stages of the endocytic pathway (Clemens and Horwitz 1995). It is thus able to evade the damaging conditions encountered in lysosomes. It has been reported that Mtb can arrest phagosome maturation through a variety of pathways including interaction with mouse coronin-1, or 'TACO' (tryptophane aspartate-containing coat protein) (Ferrari *et al.*, 1999). TACO is a host protein present on lymphoid and myeloid cells, which presents on the surface of phagosomes containing Mtb, and blocks their maturation into phagolysosomes. It was suggested that live bacteria were able to interact with and retain TACO on the phagosomal membrane. Release of TACO was thought to be the trigger for phagosome fusion. More recently, it has been demonstrated using human macrophages, that human coronin-1 is only

associated with early stages of mycobacterial phagocytosis and the protein is released from the phagosomal membrane soon after the bacteria are engulfed by the macrophage (Schuller *et al.*, 2001). However, evidence does seem to support a role for an as yet unidentified protein in the inhibition of phagosome maturation.

Mtb can also control phagosome acidification, to prevent the activation of hydrolases, which promote destruction of phagocytosed bacteria (Dubnau and Smith 2003). The suggested mechanism is thought to be due to exclusion of the vesicular proton ATPase (Sturgill-Koszycki *et al.*, 1994). A further survival mechanism is the ability of Mtb infected macrophages to evade apoptosis and it has been shown that more virulent mycobacterial strains are less likely to undergo apoptosis in macrophages (Keane *et al.*, 2000).

Therefore, Mtb have evolved a range of mechanisms to manipulate their surroundings and decrease the effects of the immune response in order to promote their intracellular survival.

1.7.2 Detoxification of RNIs and ROIs

Mtb possesses a variety of mechanisms to counter the harmful effects of antimicrobial agents produced by macrophages and detoxify reactive oxygen and nitrogen intermediates.

These include the *katG* gene, which encodes the only catalase/ hydroperoxidase in Mtb. The correlation between loss of *katG* activity and virulence is controversial, but it is postulated that it plays a role in resistance to hydrogen peroxide *in vitro*

and oxidative stress *in vivo* (Manca *et al.*, 1999). Mutations in *katG* are associated with isoniazid resistance (Zhang *et al.*, 1992).

The gene *ahpC* encodes a subunit of the enzyme alkyl hydroperoxide reductase which has been implicated in decreasing the toxicity of organic peroxides (Niimura *et al.*, 1995), and peroxynitrite (Bryk *et al.*, 2000). In other mycobacterial species, expression of *ahpC* is regulated by the *oxyR* gene which in *Mtb*, is an inactivated pseudogene containing a number of lesions (Deretic *et al.*, 1995; Sherman *et al.*, 1995), and therefore dysfunctional. *oxyR* is a hydrogen peroxide response activator which can both sense oxidative stress and activate the response to it. In *E. coli* detoxifying genes including *katG* and *gorA*, a glutathione oxidoreductase are regulated by *oxyR* in addition to *ahpC*. Loss of the *oxyR* gene and, therefore, its regulatory effects on *ahpC* have been implicated in isoniazid resistance (Deretic *et al.*, 1995). Despite this, it has been found that *ahpC* is not required for virulence using a mouse model of infection (Springer *et al.*, 2001). Nonetheless, gene expression is elevated in certain phases of *in vitro* growth and an *ahpC* mutant was shown to be more susceptible to killing by organic peroxides (Springer *et al.*, 2001). This is therefore a potentially important gene in surviving the immune response although its exact role in virulence remains unclear.

In *E. coli*, the SoxRS gene products regulate the transcription of superoxide dismutases, SodA and SodC. These enzymes play an important role in dismuting toxic superoxide radicals into hydrogen peroxide and oxygen (Dussurget *et al.*, 2001) and homologues of these genes and this system are found in *Mtb*.

SodA is a MnFe superoxide dismutase and is a major extracellular protein in Mtb. However, its role is largely unknown although it is thought that it is involved in protection against superoxide during early infection in mice (Piddington *et al.*, 2001). SodC is a CuZn superoxide dismutase and is produced at a much lower level than SodA. However, SodC is thought to protect Mtb against extracellular superoxide generated by host cells, and is implicated in survival following exposure to superoxide and the oxidative burst generated by macrophages activated with IFN- γ (Piddington *et al.*, 2001). Additionally, a *sodC* mutant in Mtb was found to be more susceptible to the toxic effects of hydrogen peroxide (Dussurget *et al.*, 2001) which supports the role of the gene in the detoxifying response to ROI. However, the role of SodC during early infection in mice appears to be limited (Piddington *et al.*, 2001).

Lipoarabinomannan (LAM) has been shown to scavenge oxygen free radicals as well as block transcriptional activation of IFN- γ in human macrophage-like cells (Chan *et al.*, 1991). Mycothiol, a low molecular weight thiol in mycobacteria, has been implicated in the protection of Mtb from oxidants. The production of mycothiol has been shown to be essential for growth of Mtb *in vitro* (Sareen *et al.*, 2003), and is thought to be necessary for growth of Mtb under intracellular conditions. The *noxR1* gene has been shown to confer resistance to ROI and RNI in *E. coli* and Msm (Stewart *et al.*, 2000). However, despite a homologue of this gene being present in Mtb, mutation of the gene has no effect on survival following exposure to RNI generated *in vitro* (Stewart *et al.*, 2000).

1.7.3 DNA Repair and Mtb survival during infection

If the damaging conditions induced by the host immune response cannot be averted or evaded, the bacteria are exposed to them and, in order to survive, must repair the damage sustained. Additionally, bacteria surviving deep within granulomas are subject to suboptimal conditions and low level assault from the host immune system. The importance of DNA repair for Mtb survival is supported by the vast range of pathways available to repair the damage and the conservation of these pathways throughout different species.

During exposure to RNI and ROI, the bacterial DNA is damaged and, therefore, must be repaired. Damage to the bacterial DNA can cause a variety of lesions and there are mechanisms available to repair almost all types of damage. The simplest form of DNA repair in Mtb involves an alkylguanine-DNA transferase, *ogt*. The enzyme encoded by this gene catalyses a simple reaction to repair a specific type of damage by directly reversing it.

It has been demonstrated in other bacteria that damage induced by ROIs and RNIs *in vivo* is repaired by the base excision repair pathway (Sancar 1994) and also by homologous recombination (Buchmeier *et al.*, 1993). However, as bacterial survival *in vivo* may depend on the ability to repair damaged DNA, it seems increasingly unlikely that only one specific pathway can repair a specific kind of damage. The major pathways for repairing damaged DNA in Mtb include Homologous Recombination, Nucleotide Excision Repair, Base Excision Repair and the recently identified Non-homologous End Joining.

1.8 Mechanisms of DNA repair

In humans, the crucial role of DNA repair is highlighted by the fact that individuals deficient in either single components or entire pathways are susceptible to the effects of otherwise benign or repairable damage. This can be demonstrated in individuals with Xeroderma pigmentosum, who are deficient in components of the nucleotide excision repair pathway (Cleaver 1968) and are exquisitely sensitive to the effects of UV radiation from sunlight. Likewise, individuals with Familial Adenomatous Polyposis Coli lack the DNA repair enzyme MYH glycosylase (Sampson *et al.*, 2003), which corrects mis-pairing via base excision repair. Without this repair system, mutations accumulate and eventually lead to tumour formation.

In intracellular bacteria, DNA repair is used for a different yet crucial role in the repair of damage sustained as a result of exposure to the human immune response.

1.8.1 Homologous Recombination

Homologous Recombination (HR) is one of the main pathways involved in the repair of double strand breaks. The pathway is of very high fidelity and involves genetic exchange between two DNA molecules with regions of homology, using one DNA molecule as a template in order to retain the integrity of the DNA sequence (Hiom 2000). HR is involved in the repair of DNA primarily during replication, where there is a homologous template.

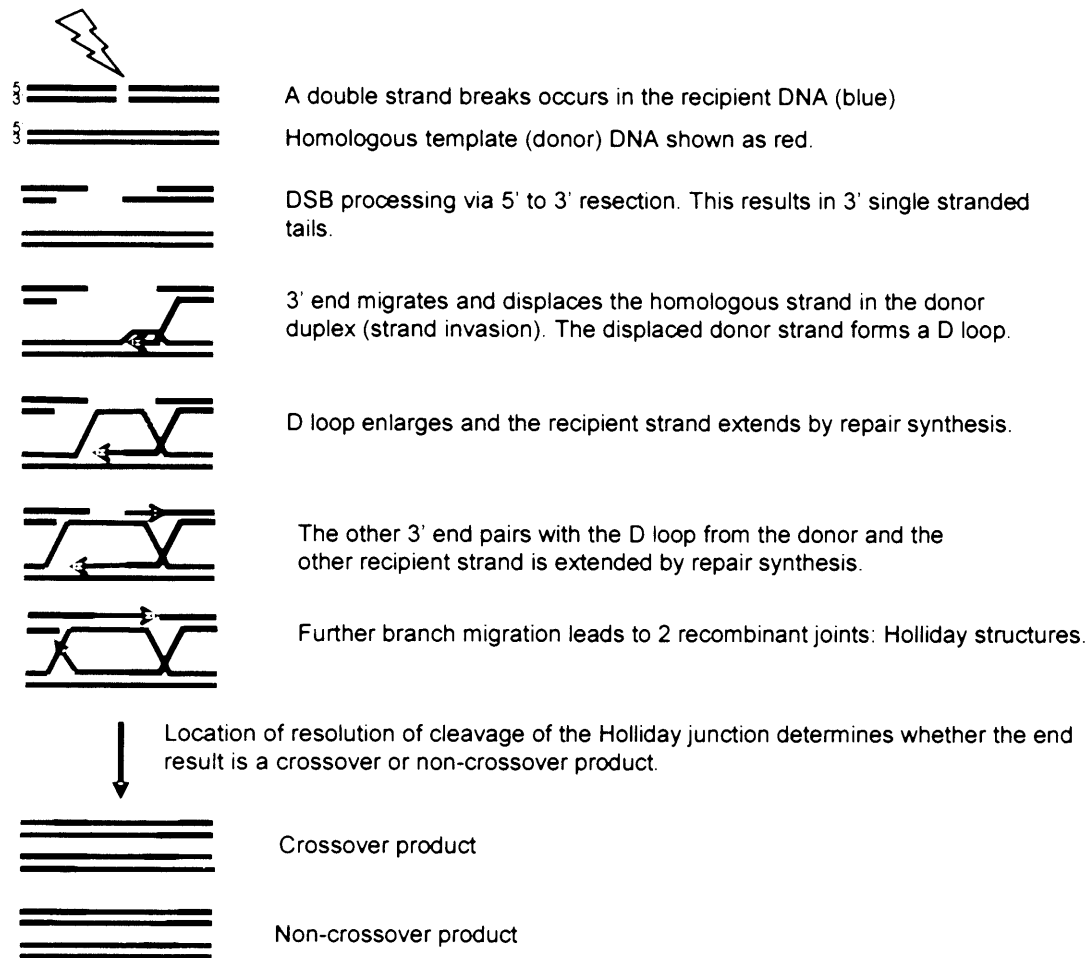
Homologous Recombination is initiated by a region of single-stranded DNA that provides the nucleation point for RecA to form a nucleoprotein filament. It is this nucleoprotein filament that is the key to the processes of homology searching and strand exchange (Wyman *et al.*, 2004). The necessary single-stranded DNA can be formed in a variety of ways and involves different additional recombination proteins depending on the nature of the initial DNA substrate.

The process can be divided into 3 stages, strand exchange, branch migration, and resolution (Hiom 2001), which occur sequentially. Initially, double strand break formation is followed by subsequent nucleolytic processing. This results in single stranded 3' ends, onto which RecA can load. The primary function of this initial stage is to create a suitable DNA substrate for the nucleoprotein filament (Wyman *et al.*, 2004). Unwinding of the DNA, nucleolytic processing and RecA loading are coordinated by the RecBCD protein complex (Kowalczykowski 2000).

A joint molecule is formed between the processed broken DNA and the intact template DNA. The RecA nucleoprotein filament then mediates the strand invasion to form a heteroduplex (Lusetti and Cox 2002). The complex formed between the two DNA duplexes is initially stabilised by hydrogen bonding and is later reinforced by covalent bonds due to enzyme mediated repair of the DNA breaks. The joint molecule is connected at the recombination joint and is referred to as the Holliday structure. The DNA at the point of contact comes from two different sources and is not identical. One strand comes from each of the parental DNA molecules. This region of heteroduplex DNA is extended by branch migration (West 2003) and involves the unpaired region of one of the single

strands of DNA displacing the paired region of the other single strand. In this way, the crossover point is able to move by branch migration. RecA induces directional migration, which can result in a heteroduplex DNA region of thousands of base pairs in one direction. Alternatively, spontaneous branch migration may occur. This involves equal migration in both directions but is more restricted in terms of the length.

Finally, in the resolution stage, the joint molecule separates into two distinct DNA molecules by structure-specific endonucleases. Resolution requires two cuts in the joined DNA molecules and the site of these nicks are important for determining whether the cutting of the strands produces a crossover or non-crossover product (Figure 1.1).



(Adapted from Atherly *et al.*, 1999)

Figure 1.1: Homologous Recombination

The figure depicts the stages involved in homologous recombination. The DNA double strand break occurs and is enlarged by exonuclease action. 5' to 3' resection occurs and single strands are left with 3' ends. A D-loop is formed via strand invasion and the recipient strand is extended by DNA synthesis. Further branch migration results in the formation of Holliday structures. The location of cleavage during resolution determines whether the product will be a crossover or non-crossover.

In bacteria there are usually two pathways for HR, the RecBCD and RecF pathways. Both of these require RecA to perform the reactions central to the process of recombination; their primary difference is in the proteins involved in the formation of the RecA nucleoprotein filament. In *E. coli* the RecBCD pathway is referred to as the major pathway and processes double strand breaks. Here, the RecBCD enzyme initiates recombination via its nuclease and helicase activities (Amundsen *et al.*, 2000) that provide the appropriate substrates for RecA to bind to, allowing homologous recombination (Chedin and Kowalczykowski 2002). Initially, unwinding from a double strand break is coupled to degradation of the 3' terminated DNA strand, but once the complex reaches and recognises a specific DNA motif termed a Chi site, its activity is modified resulting in degradation of the 5' strand and the formation of a 3' single-stranded DNA substrate onto which RecA is loaded (Anderson and Kowalczykowski 1997).

Interestingly, homologues to the RecBCD genes are found in only a limited number of bacteria which implies a relatively recent evolution in bacteria (Eisen and Hanawalt 1999). In particular, these genes are not found in most Gram-positive bacteria although equivalent functions are provided by unrelated proteins termed AddAB in a number of cases (Chedin and Kowalczykowski 2002). The AddAB enzyme in *B. subtilis* possesses ATP-dependent helicase and nuclease activities, which allows double stranded DNA to unwind and degrade during translocation. AddAB recognises and responds to the Chi DNA sequence by forming a stable complex with it (Chédin *et al.*, 2006). However, RecBCD homologues are present in Mtb.

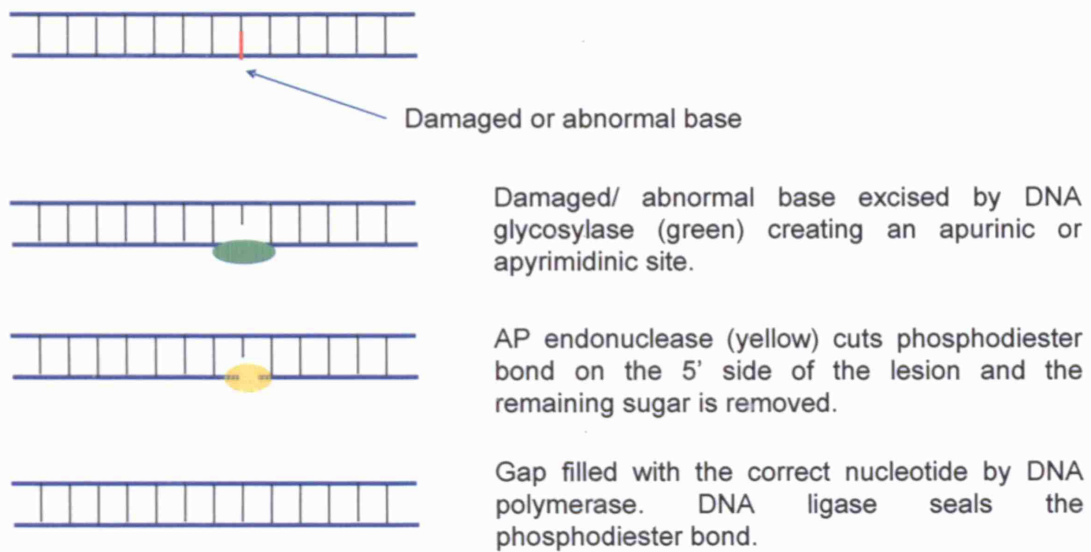
The minor recombination repair pathway in *E. coli* involves the *recFOR* genes and is thought to play a role in the reactivation of stalled replication forks and the repair of single strand breaks (Friedberg *et al.*, 1995). Although the precise functions of these proteins are not yet clear, they have been shown to be important for loading RecA onto gapped DNA coated with single-stranded binding protein (Morimatsu and Kowalczykowski 2003). The RecFOR proteins also appear to contribute to protection of the nascent lagging strand of arrested replication forks (Chow and Courcelle 2004). Although the RecFOR pathway was thought of as the minor pathway in *E. coli*, the *recF*, *recO* and *recR* genes are more widespread amongst bacteria than the *recBCD* genes, suggesting that they perform an important function. All three genes are thought to be present in Mtb (Mizrahi and Andersen 1998; Muniyappa *et al.*, 2000). In contrast, mycobacteria lack homologues of the exonuclease RecJ or the helicase RecQ, which can allow the RecFOR pathway to act on double strand breaks in *E. coli*.

recA mutant strains in *E. coli* show a marked decrease in recombination efficiency compared with the wild type strain (Kowalczykowski *et al.*, 1994), confirming the key role of RecA in this process. It has also been demonstrated that both a *recA* mutant strain and a *recBCD* mutant strain in *S. typhimurium* showed decreased survival in macrophages (Buchmeier *et al.*, 1993) and were highly attenuated in mice (Cano *et al.*, 2002), confirming the importance of the pathway, and also of DNA repair in general, for survival *in vivo*. As well as being able to repair double strand breaks, HR can also repair other DNA lesions such as inter-strand crosslinks, thus broadening the range of DNA damage it can repair.

As mentioned previously, Holliday junctions are formed when HR is initiated at DNA ends and single strands are exchanged between homologous partners, causing the formation of 4 way branched intermediates. RuvC is a Holliday junction endonuclease and acts with RuvAB to resolve Holliday junctions (West 2003). All three of these genes are present in Mtb. It has been found that *Helicobacter pylori* *ruvC* mutant strains show increased susceptibility to oxidative stress and are attenuated in macrophages (Loughlin *et al.*, 2003). This demonstrates that other components of the HR system, as well as RecA are important in repairing damaged DNA.

1.8.2 Base Excision Repair

Base Excision Repair (BER) is an important pathway in DNA damage repair as it repairs damage caused by exposure to metabolites which oxidise and alkylate DNA (Mizrahi and Andersen 1998), resulting in incorrect base pairing. BER is a two-stage process. In the first step a damaged or abnormal base is excised by a DNA glycosylase, such as MutY or Nth, creating an apurinic or apyrimidinic site. In the second stage of the pathway, an apurinic/apyrimidinic (AP) endonuclease, such as XthA, cuts the phosphodiester bond immediately on the 5' side of the AP site and the remaining sugar is removed. Following these specific processes, the gap is filled with the correct nucleotide by DNA polymerase and DNA ligase seals the phosphodiester bond (Figure 1.2).



(Adapted from Augusto-Pinto *et al.*, 2003)

Figure 1.2: Base Excision Repair

The schematic demonstrates the basic stages of base excision repair. Once the damaged base is identified, it is excised by a DNA glycosylase such as MutY, or Nth, causing the formation of an apurinic or apyrimidinic (AP) site. Subsequently, an AP endonuclease, such as XthA cuts the phosphodiester bond and the remaining sugar is removed. This then allows the gap to be filled with the correct nucleotide via DNA polymerase. The gap is sealed by DNA ligase (Augusto-Pinto *et al.*, 2003). Base excision repair is implicated in the repair of damage sustained as a result of DNA oxidation and alkylation (Mizrahi and Andersen 1998).

There are a number of DNA glycosylases which specifically recognise different kinds of damaged bases. The Fpg-Nei family of proteins function to repair damage arising from exposure to ROI (Cabrera *et al.*, 1988). The Fpg protein recognises oxidised residues such as 8-oxo-G (8-oxo-7,8-dihydroguanine), an oxidation product of guanine (Dempfle and Harrison 1994). 8-oxo-G is highly mutagenic due to its mispairing with adenine during replication, resulting in G to T transversions. Nei is a homologue of Fpg (Duwat *et al.*, 1995) with similar function but with different substrate specificity, such as thymine glycol, thus widening the diversity of the type of lesion which can be repaired. MutY is an adenine glycosylase that primarily removes A if it has been incorporated opposite 8-oxo-G, allowing the incorporation of the correct nucleotide in a second round of synthesis and another opportunity for the removal of the 8-oxo-G by Fpg. Ung (uracil DNA glycosylase) is specific for the removal of uracil from DNA; this can arise as a consequence of deamination of cytosine, for example by RNI. Homologues of all these glycosylases can be identified in Mtb.

There are two classes of AP endonucleases, represented in *E. coli* by *xthA* (exonuclease III) and *nfo* (endonuclease IV), although these both cleave the DNA at sites of base loss in the same way to leave 3' hydroxyl groups suitable for extension by polymerases. Mtb possesses homologues of both *xthA* and *nfo*, although the homologue of *nfo* has been annotated as *end* (Cole *et al.*, 1998). In *E. coli*, mutants lacking exonuclease III, the gene product of *xthA*, are killed by DNA damage induced by hydrogen peroxide (Galhardo *et al.*, 2005), and BER mutants in *S. typhimurium* have been found to be severely attenuated in a macrophage model of infection, a phenotype that reverted to wild type levels in macrophages

unable to form an oxidative and nitrosative stress response (Suvarnapunya *et al.*, 2003). These findings highlight the importance of the role of BER in repair of DNA damage arising from exposure to activated macrophages.

Mismatch repair, using the *mutSLH* repair pathway, is a specialised form of base excision repair which is found in a wide variety of bacterial species and is well characterised in *E. coli*. The repair is specific to the newly synthesised strand of DNA, recognised in *E. coli* via its methylation status. However, homologues of the proteins involved have not been identified in Mtb (Mizrahi and Andersen 1998) and the pathway has been demonstrated to be lacking in mycobacteria (Springer *et al.*, 2004).

1.8.3 Nucleotide Excision Repair

Nucleotide excision repair (NER) involves the incision of the damaged strand on the 3' and 5' sides of the lesion, followed by the removal of an oligonucleotide containing the damaged base. The stages are commonly split into 4 distinct parts: (i) initial recognition of the damage and verification of the lesion type, (ii) incision, (iii) displacement of the damaged oligonucleotide, and (iv) repair synthesis and ligation. The process of NER is highly conserved from *E. coli* to humans, although there is some variation in the proteins involved.

In humans, nucleotide excision repair is especially important for repairing damage sustained by exposure to UV radiation from sunlight. This DNA damage is most often in the form of thymine dimers and (6-4) photoproducts. Inability to repair this type of damage can result in cancer as is demonstrated in individuals with

xeroderma pigmentosum and Cockayne's syndrome, both caused by mutations in components of NER.

Although the pathway is similar in eukaryotes and prokaryotes, as would be expected it is more complex in higher organisms and involves a greater number of components. The removed oligomer in humans is longer than that removed in *E. coli* and consists of around 24-32 nucleotides, double that removed in *E. coli*. However, the general principle is the same. For human NER, 6 repair factors are required to remove the damaged DNA. These are XPA, RPA, XPC, TFIIH, XPG and XPF-ERCC1. XPA, RPA and XPC locate the damage and recruit TFIIH, a transcription/ repair factor. TFIIH contains 6 polypeptides, which unwind the DNA around the damaged site. XPG incises on the 3' side and XPF-ERCC1 incises on the 5' side of the lesion. The resulting gap in the DNA strand is filled by repair synthesis proteins, replication factor C, proliferating cell nuclear antigen and DNA polymerases δ and ϵ . Finally, the repaired DNA is sealed by DNA ligase I (Mu *et al.*, 1996; Hutsell and Sancar 2005)

In bacteria, the pathway is simpler and has been well characterised in *E. coli*. It involves a group of genes, *uvrABC* and *D*. The UvrA and B proteins function together to locate the damaged DNA. They form a damage recognition complex which is comprised of 2 subunits of each of UvrA and UvrB (Verhoeven *et al.*, 2002). This damage recognition complex has a higher affinity for damaged DNA, compared with intact DNA (Sancar and Sancar 1988). The UvrA subunit recognises distortions in the DNA helix and UvrB unwinds a short segment of DNA to separate the two DNA strands. UvrB can bind to a plethora of structurally

unrelated lesions and it has been demonstrated that a β -hairpin structure is involved in damage specific binding (Malta *et al.*, 2006). The presence of two subunits of UvrB allows both DNA strands to be checked for damage. The mechanism is such that the DNA wraps itself around the first UvrB monomer (Verhoeven *et al.*, 2001) and is scanned for damage. If no damage is found, the DNA wraps around the 2nd UvrB subunit and the process repeats (Verhoeven *et al.*, 2002).

In the presence of damage to the DNA strand, UvrC binds to UvrB, making a UvrBC DNA incision complex and one of the UvrB monomers is released (Verhoeven *et al.*, 2002). In order to specifically incise the damaged strand, 3' incision by UvrC requires the presence of a UvrB/damage verification complex (Theis *et al.*, 1999), and interaction between the C-terminal domain of UvrB and a homologous region in UvrC (Moolenaar *et al.*, 1995). Two incisions are made by UvrC, which consists of 2 functional halves, an N-terminal domain which makes the 3' incision 4-5 nucleotides away from the damaged base and a C-terminal domain which makes the 5' incision (Verhoeven *et al.*, 2002). The C-terminal region of UvrC, is homologous to the C-terminal region of the ERCC1 protein (Westerveld *et al.*, 1984; Moolenaar *et al.*, 1998), the component in human NER which is involved in DNA binding and 5' incision. The 5' incision is made approximately 8 nucleotides away from the damage. The second cut is about 1 turn of the helix from the first. This results in an excised single stranded segment of DNA, which is approximately 12 nucleotides long. The helicase UvrD, displaces the released oligonucleotide with UvrC, allowing repair via DNA polymerase I and ligase, which seals the newly repaired segment at the 3' end

(Asad *et al.*, 1995; SaiSree *et al.*, 2000). All the components required for NER are present in Mtb and the ability to repair damage sustained as a result of exposure to UV light is presumably an important attribute for Mtb, as it must be able to withstand this stress if it is to be able to spread effectively from person to person.

In *E. coli*, a protein termed Mfd targets NER to the transcribed strand of actively transcribing genes in a sub-pathway called transcription coupled repair (Selby and Sancar 1995). Mfd also has a homologue in Mtb.

Recently, a new addition to the NER pathway has been identified in *E. coli* (Moolenaar *et al.*, 2002). It was discovered that there was an additional incision enzyme, homologous to the N-terminal part of UvrC. Hence, the protein was named Cho, UvrC homologue. The pathway is shown as a schematic in figure 1.3, incorporating the role of Cho.

UvrA and B locate the damage on the strand, demonstrated here by a thymine dimer. The DNA wraps around the UvrB subunit. In the event of damage, UvrB remains as a dimer.

UvrC and Cho are recruited to the damaged strand where they incise DNA on the 5' and 3' sides of the lesion respectively. Upon binding to UvrC, one of the UvrB subunits is released.

UvrD mediates the release of UvrB+C and Cho. DNA polymerase I and DNA ligase complete the repair.

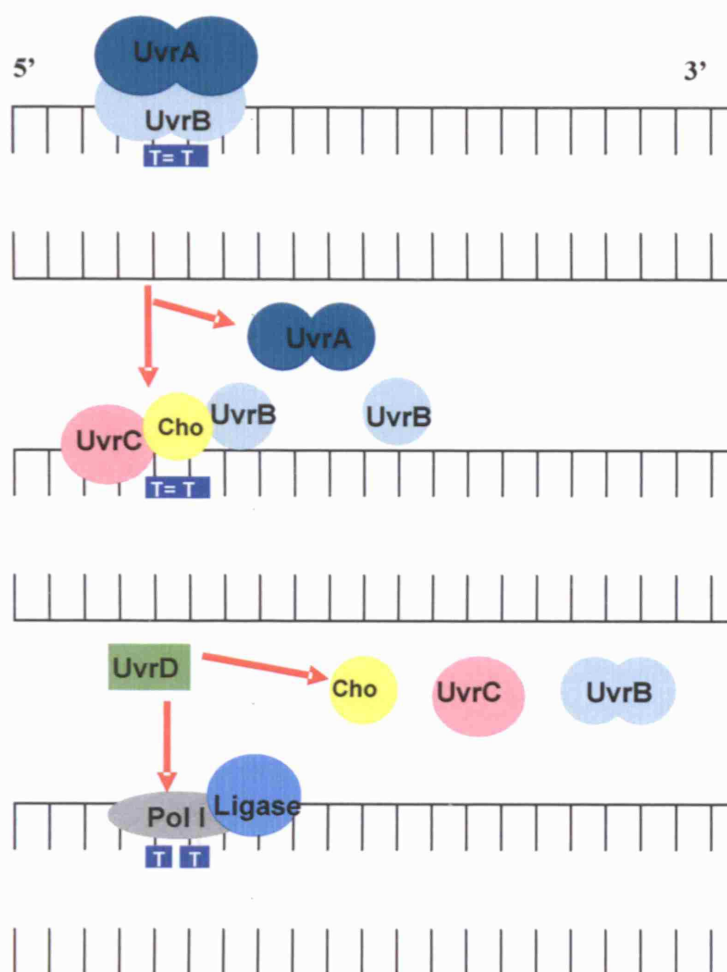


Figure 1.3: Nucleotide Excision Repair in *E. coli*

A schematic diagram of NER processing of damage, incorporating a potential role for Cho. The UvrA and UvrB protein complex locates the damage and recruits UvrC and Cho for the incision stage. These proteins bind to UvrB at different positions and incise the damaged DNA. The complex is removed via UvrD, which then recruits PolI and ligase to seal the damaged DNA at the 3' end.

The gene coding for Cho, *ydgQ*, in *E. coli*, is induced by DNA damage (Lewis *et al.*, 1994) and encodes a 295 amino acid protein. Interestingly, Cho has a number of functions which are complementary to those of UvrC. Purified Cho produces incisions on the 3' side of the lesion only. This occurs at the ninth phosphodiester bond, 3' to the damaged nucleotide and is a further 4 nucleotides away from the site that UvrC would normally incise (Moolenaar *et al.*, 2002). The 5' incision is then made by UvrC. This results in a bigger segment of excised DNA than that formed by UvrC alone, which suggests a role for the removal of bulkier lesions.

Biochemical assays have shown that specific damage which is poorly incised by UvrC alone, can be efficiently incised by Cho (Moolenaar *et al.*, 2002). This evidence implies that for certain types of lesion Cho plays a potentially important role. Like UvrC, Cho is dependent on the presence of a UvrB damage verification complex (Theis *et al.*, 1999) but does not require the UvrC binding domain on the UvrB protein (Moolenaar *et al.*, 2002).

A DNA damage inducible gene homologous to Cho has been identified in Mtb (Moolenaar *et al.*, 2002). It is termed Rv2191. Interestingly, this gene is larger than its *E. coli* homologue, and contains an extra domain, which has strong elements of homology to a 3' exonuclease, the proof reading subunit of the DNA polymerase III holoenzyme. This could suggest that Rv2191 may display exonuclease activity linked to the 3' incision activity (Moolenaar *et al.*, 2002). It also leads to questions surrounding the role of NER in Mtb and why there should be a difference between the two bacterial homologues of Cho. It is tempting to speculate that the additional domain and its extra capabilities may be somehow

linked to either the pathogenic nature of Mtb or to some aspect of its survival, either during transmission or during intracellular infection.

Although there has been some biochemical characterisation of Cho in bacteria (Moolenaar *et al.*, 2002), there is limited information about the role of Cho (Rv2191) in Mtb. Other components of NER have been characterised to an extent (Darwin and Nathan 2005) but so far, there have been no investigations into the role of Rv2191. Although biochemical investigations provide a good idea of function *in vitro*, it is difficult to fully characterise the role of the gene in this way. Constructing mutant strains are a good way to further investigate a specific gene and examine the role of the missing gene both *in vitro* and whenever possible, *in vivo*.

1.8.4 Non-Homologous End Joining

Non-homologous end joining (NHEJ) is an alternative repair pathway used to repair double strand breaks (DSBs). The broken ends are simply re-ligated back together without the need for a homologous template as is the case in homologous recombination. As a consequence, a small number of bases can be lost or inserted at the repair site and the integrity of the DNA can be compromised at the expense of successful repair. NHEJ appears to be the primary mechanism for DSB repair in mammalian cells (Jeggo 1998), probably because the mechanism is active throughout the cell cycle (Critchlow and Jackson 1998). However, homologous recombination appears to be the main mechanism of DNA repair in lower eukaryotes such as *Saccharomyces cerevisiae* (Dudášová *et al.*, 2004).

For successful homologous recombination to take place, extensive sequences of homology are required, whereas for NHEJ only a few homologous base pairs are necessary; these are known as microhomologies and are used to guide the repair. Where double strand breaks are present in the form of single stranded overhangs, the microhomologies are used to create accurate repair with no sequence loss. Where there is no region of homology present, the ends are simply religated back together, leading to potential introduction of error. However, this is rare and on the whole, the pathway is extremely accurate.

NHEJ is conserved throughout many species and is present from higher organisms, including humans, through to yeast. In addition, this repair pathway has recently been identified in prokaryotes such as *Mtb* and *Bacillus subtilis*. Interestingly, *E. coli* does not have a NHEJ system.

1.8.4.1 NHEJ in eukaryotes and higher organisms

In humans, NHEJ is used primarily for the repair of DSBs arising as a result of exposure to ionizing radiation, certain chemicals and following V(D)J chain rearrangement (Hiom 2003), the process whereby B cell and T cell receptor diversity is generated in vertebrate cells. The pathway is particularly important as unrepaired DNA double strand breaks can result in catastrophic consequences for the cell including death, cell cycle arrest and chromosome translocation, which can result in increased mutation rates, ultimately leading to tumour formation.

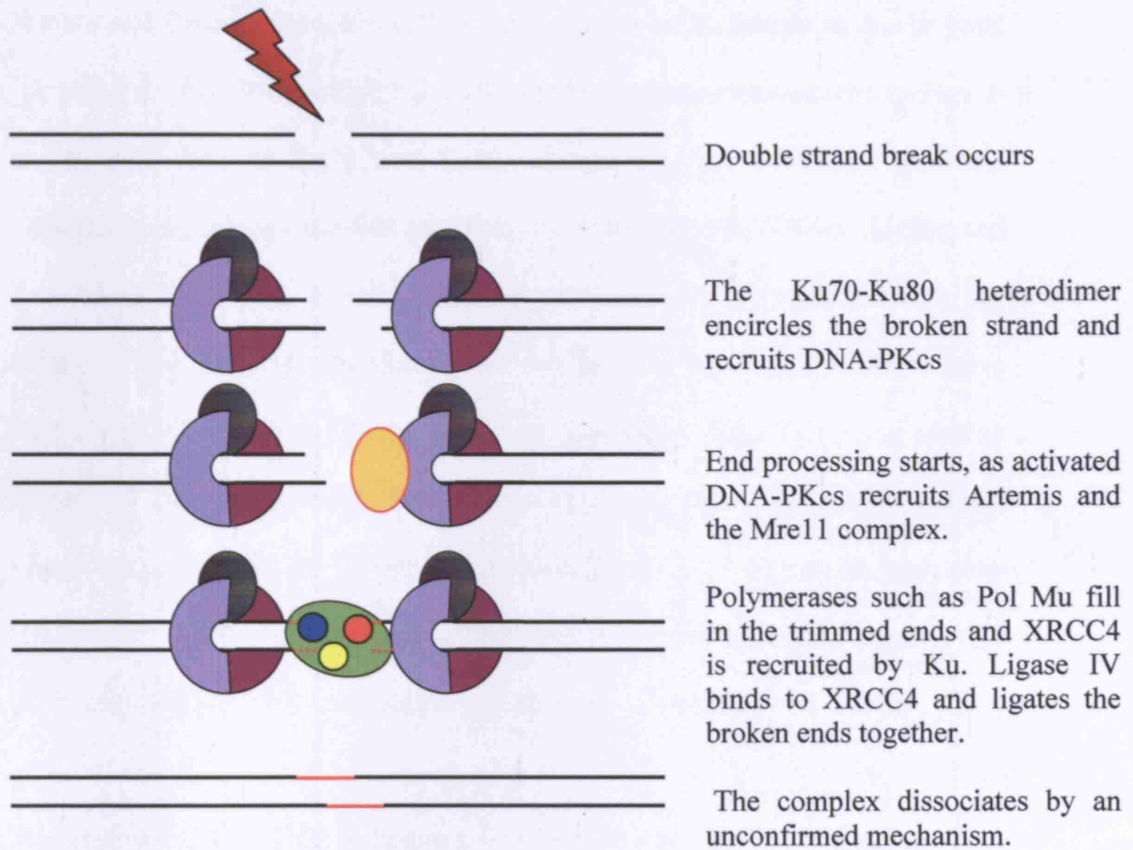
The pathway in higher organisms and eukaryotes involves a number of components, which make up a multi-protein complex, sometimes referred to as

DNA-PK. The complex includes a DNA-dependent protein kinase catalytic subunit, (DNA-PKcs), (Smith and Jackson 1999), a heterodimer consisting of Ku70 and Ku80, XRCC4 and Artemis, as well as some other factors, which are as yet unidentified (Roth 2003).

The main function of the Ku70/Ku80 heterodimer is recognition of DSBs. It has been demonstrated *in vitro* that the Ku heterodimer has a high affinity for a variety of DNA ends as well as hairpins which occur as intermediates during V(D)J chain rearrangement (Dylan and Yoo 1998).

The Ku heterodimer recruits the DNA-PKcs. The DNA-PK becomes activated during association with the Ku70/Ku80 heterodimer bound to DNA and preferentially phosphorylates proteins bound on the DNA (Doherty *et al.*, 2001). Together the Ku/DNA-PKcs complex loads on to the broken DNA strand, binding to the ends and forming a ring around it. The Ku proteins arrange themselves into a hollow circular structure through which DNA ends can be threaded allowing repair (Doherty and Jackson 2001). The Ku/DNA-PKcs complex diffuses along the DNA. Activated DNA-PKcs recruits Artemis, an endonuclease, and Mre11 to process the ends. Pol Mu (polymerase) fills in the gap in the DNA strand and XRCC4 is recruited by Ku. The DNA ligase IV (an ATP-dependent DNA ligase) binds to XRCC4 and joins the ends together, the components then dissociate (Figure 1.4). It is currently unclear exactly how the repair complex disassembles from ligated DNA.

Interestingly, eukaryotic Ku proteins have a range of functions due to their additional domains, which are thought to aid their role in DNA repair (Doherty and Jackson 2001). These include a Von Willebrandt factor A domain (Ponting *et al.*, 2000), which enables Ku to recruit additional proteins to the site of damage, and a SAP DNA binding motif (Aravind and Koonin 2001). The SAP motif is thought to prevent Ku from moving away from the DSB ends (Walker *et al.*, 2001).



Adapted from Weller *et al.*, 2004

Figure 1.4: Non-homologous end joining in eukaryotes

The process of NHEJ is relatively complex in eukaryotes and involves a number of components. There remains the possibility that further components have yet to be identified. The Ku70-Ku80 heterodimer forms a ring around the broken strand and recruits Artemis and the Mre11 complex and end processing starts. Polymerases fill in the trimmed end and Ligase IV ligates the broken ends together. This is followed by dissociation of the components.

The eukaryotic Ku homologues are of similar sizes and subunit structure across the species and display similar DNA binding properties (Dynan and Yoo 1998). Human and *Drosophila* Ku70 cDNA can complement *ku* mutant strains in yeast (Dynan and Yoo 1998), which highlights the conservation between the species. It is proposed that the Ku70 and Ku80 subunits are derived from a common ancestral gene, despite the fact that they are biochemically distinct (Dynan and Yoo 1998; Gell and Jackson 1999). Additionally, it is suggested that the eukaryotic and bacterial Ku proteins are derived from a common ancestral gene, which probably bound to DNA ends and recruited other factors for repair such as ligases (Doherty *et al.*, 2001). This is supported by the ability of bacterial Ku and Ligase to complement the joining deficient phenotype of NHEJ mutant yeast cells (Della *et al.*, 2004). However, the reverse does not hold true, which suggests that in prokaryotes, the NHEJ complex is species specific (Weller *et al.*, 2002).

The importance of NHEJ in humans is highlighted by the fact that defects in a number of its components are linked to cancer and immune related deficiencies. Mice deficient in DNA-PKcs are severely immunodeficient, due to the inability to carry out successful V(D)J chain rearrangement and additionally are sensitive to ionizing radiation. Ku70 and Ku80 knockout mice display the severe combined immunodeficiency (SCID) phenotype (Barnes 2001) for the same reason. Individuals with mutations in the gene coding for DNA ligase IV have been demonstrated to be immunodeficient and additionally display developmental and growth defects. It seems that the breadth of function of NHEJ and its components is huge and for this reason, not yet fully understood.

1.8.4.2 NHEJ in prokaryotes

The pathway of NHEJ in bacteria was discovered only recently in *Bacillus subtilis* (Weller *et al.*, 2002). The process is predicted to be simpler in prokaryotes than eukaryotes. Interestingly, *ku* genes are not present in all bacterial species and as there is no clear phylogenetic link between bacteria that possess *ku* and those that do not, it is suggested that the NHEJ pathway may have been acquired by horizontal gene transfer events (Bowater and Doherty 2006).

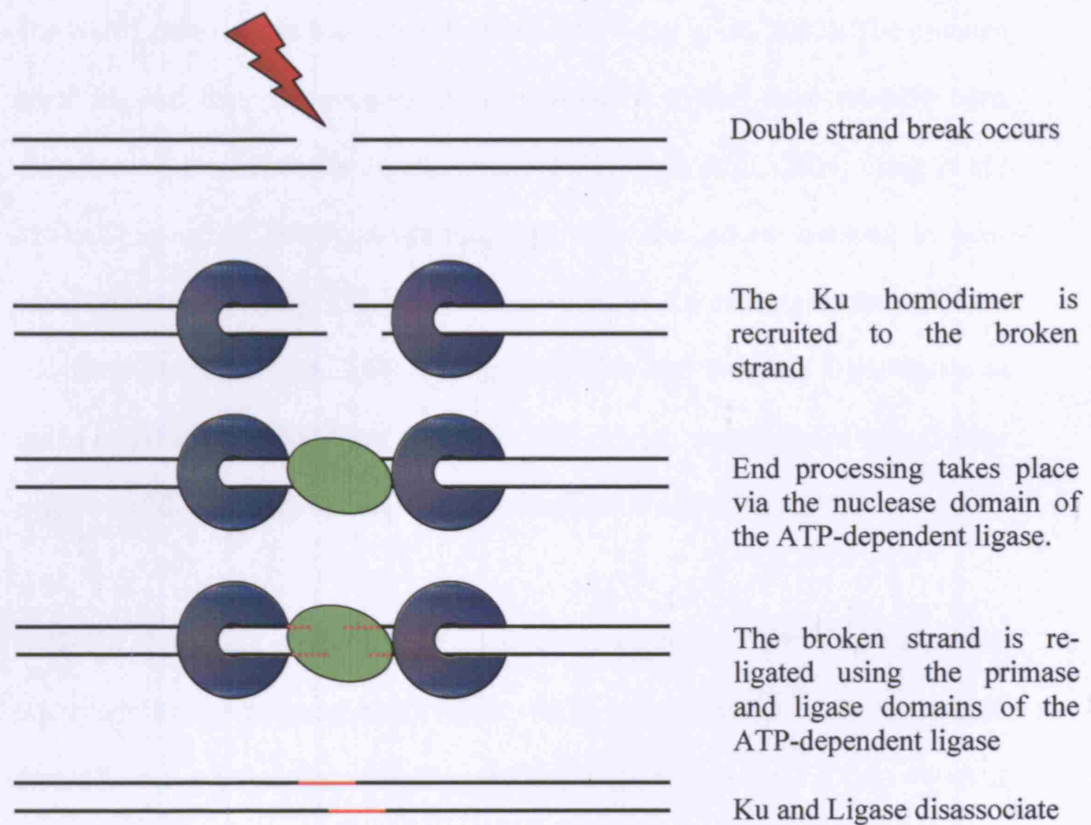
The Ku component of the pathway exists as a smaller single protein in bacteria, compared with a heterodimer in eukaryotes. However, this single protein displays homology to the core domain of both Ku70 and Ku80 (Weller *et al.*, 2002). Furthermore, it was found that in *B. subtilis*, the *ku* equivalent gene is in the same operon as the *ligase* equivalent gene. This strongly implies a functioning NHEJ pathway in bacteria (Weller *et al.*, 2002).

A protein homologous to Ku has also been identified in the bacteriophage mu. The Gam protein is similar both in sequence and structure (d'Adda di Fagagna *et al.*, 2003), and is present in a variety of bacteria. It is thought to result from prophage insertions. Its role is thought to be similar to that of Ku and following viral infection, it binds to the ends of linear phage DNA, thus preventing degradation by host exonucleases. In this way, integration of the phage genome into the host chromosome is aided (d'Adda di Fagagna *et al.*, 2003). Bacterial strains expressing *gam* or possessing Ku orthologues are enhanced in their ability to acquire DNA and integrate it into their genome (d'Adda di Fagagna *et al.*,

2003). Thus, it is suggested that Ku may have a role in prokaryotic evolution (Bowater and Doherty 2006).

NHEJ in bacteria involves the Ku-like protein and ATP-dependent DNA ligase only. On recognition of DNA ends, the Ku-like protein is recruited and loads onto the broken piece of DNA. Ku recruits the ATP-dependent DNA ligase which joins together the DNA ends and thus physically interacts both with the DNA ligase and the DNA ends. The proteins dissociate once repair is complete (Figure 1.5).

The ability of Ku to facilitate repair via DNA ligase is somewhat dependent on the type of DNA end (Ramsden and Gellert 1998). Although Ku is especially efficient when involved in the joining of blunt DNA ends, and ends with a 1 or 2 base overhang, the effect is less marked on DNA strands with a longer overhang (Hiom 2003). Ku itself does not have a role in processing the DNA strand breaks. Instead, its main role is to recruit the Ligase and potentially manage the repair in terms of order and extent (Della *et al.*, 2004). To compensate for the lack of additional processing factors, which are not present in prokaryotes, there are a number of domains with end-processing activities encoded by the *ku*-associated *ligase* genes, including a polymerase and components implicated in gap filling during NHEJ (Bowater and Doherty 2006).



Adapted from Weller *et al.*, 2004

Figure 1.5: Non homologous end joining in prokaryotes

The system of NHEJ is much simpler in prokaryotes compared with eukaryotes and involves only 2 proteins, Ku and ATP-dependent Ligase. The different domains of the ATP-dependent Ligase are used to fulfil a number of functions in the repair of the damaged strand. Once Ku is recruited to the damaged strand, end processing and religation are carried out by the ATP-dependent Ligase. Once completed, Ku and Ligase dissociate.

The NHEJ pathway has been identified in Mtb (Weller *et al.*, 2002). The proteins involved, and their homologues in *M. smegmatis* (Msm) have recently been characterised biochemically (Weller *et al.*, 2002; Della *et al.*, 2004; Gong *et al.*, 2004; Gong *et al.*, 2005), confirming that they are indeed involved in non-homologous end joining. The proteins equivalent to Ku and Ligase are Rv0937c and Rv0938 respectively. The fact that Rv0937c and Rv0938, homologous to genes in eukaryotic NHEJ, are present in Mtb strongly suggests that the pathway may be an alternative mechanism for repairing DNA double strand breaks in Mtb.

Rv0937c (Mtb Ku), shows a high level of homology to Ku70 and Ku80. The eukaryotic Ku has a central core region with N and C terminal extensions, whilst Rv0937c has a conserved core domain, Ku78 (Doherty *et al.*, 2001), which is homologous to both the Ku70 and Ku80 domains.

Rv0938, which is divergently transcribed from Rv0937c, shows significant homology to an ATP-dependent DNA ligase, which is involved in the NHEJ pathway. In eukaryotes, the ATP-dependent ligase consists of a catalytic core ligase domain flanked by N and C terminal extensions. Mtb Rv0938 is predicted to have a primase domain, a ligase domain and a nuclease domain in the centre (Weller and Doherty 2001). Interestingly, there are 4 DNA ligases in mycobacteria, Mt-ligA to Mt-ligD. Rv0938 (*ligD*), is one of the ATP dependent *ligases* and is adjacent to and divergently transcribed from *ku*. It has been demonstrated that Rv0938 displays a variety of functions, including gap-filling polymerase, terminal transferase, primase and additionally acts as a 3' to 5' exonuclease (Della *et al.*, 2004). It was thus confirmed through biochemical

studies, that with the exception of 5' digestion, Rv0937c and Rv0938 are able to undertake all the various processes of NHEJ and act as a self sufficient repair engine for DSBs (Della *et al.*, 2004). In short, Ku and Ligase together, have the ability to recognize double strand breaks, process them and ligate them together. This is in contrast to the NHEJ system in eukaryotes, which is more complex, and involves many more components (Della *et al.*, 2004).

It has been demonstrated in *M. smegmatis* that the repair of blunt and complementary 5' overhangs is highly mutagenic with a 50% error rate (Gong *et al.*, 2005). This raises some interesting questions about the purpose of NHEJ in a pathogenic organism such as Mtb, and whether this system plays a role in virulence and potentially contributes to antibiotic resistance. Biochemical studies have demonstrated that Msm LigD displays both template dependent and independent polymerase functions *in vitro* (Gong *et al.*, 2005).

As NHEJ is important in the repair of DNA damage sustained during periods of absent or decreased DNA replication where there is no homologous donor, it is tempting to speculate that this may be the primary form of repair of DSBs employed by an organism such as Mtb whilst in latency (Weller *et al.*, 2002). This is further supported by the fact that the pathway is present in *B. subtilis*, a spore former, which also spends extended periods of time in a dormant phase, whilst still maintaining its genomic integrity. *B. subtilis* NHEJ mutant spores display increased sensitivity to ionizing radiation compared with a wild type strain (Weller *et al.*, 2002), which implicates the pathway in the repair of double strand

breaks. Spores contain a single copy of their genome, which excludes the possibility of using HR for repair (Bowater and Doherty 2006).

As yet, there are no detailed studies into NHEJ and its components in *Mtb*, although biochemical analyses have been undertaken. The construction of NHEJ mutant strains would provide an insight into both the roles of the genes and the role of the pathway under various conditions

1.9 The response to DNA damage

The SOS response, an important feature in the regulation of DNA repair genes in bacteria, is induced upon exposure to a variety of DNA damaging agents. The SOS response has been well characterised in *E. coli*, where more than 40 genes are regulated in this way and are induced on exposure to DNA damage. The SOS response is regulated by RecA and LexA (Little and Mount 1982), with LexA acting as a transcriptional repressor.

LexA binds to an SOS box sequence upstream of genes it regulates and inhibits their transcription (Little *et al.*, 1981). Following DNA damage, the RecA protein becomes activated by binding to regions of single-stranded DNA, and stimulates the autocatalytic cleavage of LexA, preventing it from acting as a transcription repressor (Little 1991) as the shorter fragments are unable to bind to the SOS box to inhibit expression (Bertrand-Burggraf *et al.*, 1987). This leads to the induction of genes that are normally repressed by LexA and which are involved in the repair of damaged DNA. In short, the SOS response is a DNA repair system, where the DNA repair proteins are repressed under normal circumstances by LexA, but are

activated during conditions of DNA damage, for example, by the presence of a lesion.

In mycobacteria, it has been shown that there are two mechanisms for inducing gene expression under DNA damaging conditions (Davis *et al.*, 2002). The SOS response is either regulated by RecA and LexA as in *E. coli*, or alternatively, DNA damage induction can be independent of RecA (Rand *et al.*, 2003). A number of damage inducible genes can be induced in the absence of RecA in Mtb and many are involved in pathways essential for DNA repair such as NER (including *uvrA*, *uvrB* and Rv2191) and BER (*xthA* and *nei*). It is possible that there are two pathways for induction in case one fails or is inhibited, when the other can induce the repair of damage that could otherwise be lethal, or that the two systems respond to different signals.

1.9.1 DNA damage inducible genes

A number of genes of unknown function are induced following DNA damage suggesting that the proteins they encode may play a role in the repair of damaged DNA. Some of these genes are regulated by LexA as part of the SOS response, described previously. However, the majority of DNA damage inducible genes in Mtb are regulated by a RecA independent mechanism (Rand *et al.*, 2003). A number of these genes have a common promoter motif (Gamulin *et al.*, 2004), suggesting that their expression may be controlled by a specific sigma factor.

To begin to investigate DNA damage inducible genes involved in the SOS response, i.e. those regulated by a RecA/LexA dependent mechanism, one such gene, Rv3395c, has been selected for further study.

Upstream of Rv3395c is an SOS box with a single mismatch from the consensus sequence that is predicted to bind LexA (Davis *et al.*, 2002; Rand *et al.*, 2003). Furthermore, Rv3395c is very highly induced under conditions of DNA damage in wild-type Mtb but is not induced in a RecA mutant strain (Rand *et al.*, 2003). Together, these observations imply that the regulation of Rv3395c is dependent on RecA and LexA.

Rv3395c shows limited similarity to RecA proteins over an alignment of 140 amino acids and Tuberculist reports a 31.45% similarity with RecA from *Thiobacillus ferrooxidans* over this short alignment. This domain is an ATPase domain which was first identified in *E. coli* RecA (Story *et al.*, 1992). This RecA-like structural domain has been linked with a mechanical function which involves using energy obtained from hydrolysis or nucleotide binding to move nucleic acids or polypeptides (Ye *et al.*, 2004). A number of genes involved in DNA repair pathways, including *recG* (Singleton *et al.*, 2001) and *uvrB* (Theis *et al.*, 1999), also contain this ATPase domain which suggests a function in DNA repair. Rv3395c has been shown to be co-transcribed with Rv3394c (Rand *et al.*, 2003), which is induced upon exposure to hydrogen peroxide and UV irradiation (Boshoff *et al.*, 2003). Rv3394c and Rv3395c are predicted to interact by the STRINGS database (<http://string.embl.de/>). Rv3394c possesses a DinP domain, characteristic of mutagenic DNA polymerases, further supporting a role for this

operon in DNA repair. Additionally, Rv3395c has been proposed as a functional counterpart of *imuA*, one of 3 genes involved in error-prone repair of DNA lesions (Galhardo *et al.*, 2005). *imuA* and *imuB*, involved in inducible mutagenesis are thought to work with *dnaE2*. The Mtb *imuB* gene is Rv3394c. In *C. crescentus*, *imuA* and *imuB* are involved in DNA damage tolerance. The system of error-prone repair and inducible mutagenesis may have important consequences for Mtb survival *in vivo*.

1.10 Hypotheses and Aims

The evidence discussed suggests that DNA repair plays a potentially crucial role in Mtb survival and perhaps virulence. Despite this, only a small amount is known about many of the genes involved. Therefore, the aim of this project will be to investigate the role of a selection of genes predicted to be involved in different aspects of DNA repair. The genes selected for study are Rv0937c and Rv0938, components of the NHEJ pathway; Rv2191, which encodes a protein homologous to *E. coli* Cho and thought to be a component in NER; and Rv3395c, a DNA damage inducible gene with some limited similarity to *recA*. It is hoped that characterising these genes will provide an insight both into their individual roles, and potentially into the roles of the pathways which they are part of. Mutant strains, where the individual gene is inactivated, will be constructed for all of the genes under study.

Hypotheses:

- 1) The genes under study are involved in DNA repair during growth *in vitro*.
This will be investigated by examining the susceptibility of the mutant strains to DNA damage.

- 2) The genes under study play a role in bacterial survival during infection *in vivo*. This will be tested by investigating the ability of the mutant strains to grow in an animal infection model.

Aims:

- To construct Mtb mutant strains deficient in the following genes:
Rv0937c, Rv0938, Rv0937c/Rv0938, Rv2191, Rv3395c
- To carry out a basic initial screen to assess the susceptibility of the mutant strains to a range of DNA damaging conditions, and identify strains which may be affected to a greater extent in DNA repair.
- To rigorously investigate the phenotypes of the selected mutant strains, both *in vitro* and *in vivo*.

2 Materials and Methods

2.1 Bacterial Strains and growth conditions

Genetic manipulations were performed using *E. coli*, strain DH5 α (Invitrogen).

The Mycobacterial strain used was *M. tuberculosis* H37Rv.

E. coli was grown in L-broth, at 37°C overnight with shaking at 250 rpm for liquid culture. L-agar was used as solid media for growth (appendix I). Antibiotics or X-gal were added to the media as appropriate, (Kanamycin 50 μ g/ml, Gentamycin 20 μ g/ml, X-gal 100 μ g/ml).

H37Rv was grown in Dubos media (Difco, appendix I), supplemented with 0.2% glycerol and 4% albumin (Beckton Dickenson). Growth conditions were at 37°C in a rolling incubator at 2 rpm, (Lee *et al.*, 1991) where doubling time is approximately 17 hours. Middlebrook 7H11 agar supplemented with 2% Dubos medium albumin (Beckton Dickenson) and antibiotic (kanamycin 25 μ g/ml, gentamycin 15 μ g/ml), and/or X-gal (100 μ g/ml) where appropriate, was used as solid media for growth. All work on H37Rv was carried out in a Category 3 containment laboratory.

2.2 Recombinant DNA techniques for gene mutation

For primer and probe sequences, see appendix II

Table 2.1: Vectors

The vectors used to make plasmids for mutant construction and complementation:

Plasmid	Relevant characteristics
pBackbone	Contains Km ^R and Amp ^R cassette. Adapted from pBluescript (Gopaul 2003)
pCR-4 blunt	Commercial vector (Invitrogen)
pUC-GM	Contains Gm ^R cassette (Schweizer 1993)
pGOAL-17	Contains SacB/LacZ cassette (Parish and Stoker 2000)
pMV261	Replicating plasmid (Stover <i>et al.</i> , 1991)
pKP-186	Plasmid for constructing complement, <i>integrase</i> ⁻ (Rickman <i>et al.</i> , 2005)
pBS-Integrase	Plasmid containing <i>integrase</i> cassette (Springer <i>et al.</i> , 2001)

2.2.1 PCR

50 ng of genomic *M. tuberculosis* DNA was used in a 50 µl PCR reaction. The other components of the reaction were 5 µl Pfu buffer (Stratagene), 5 µl 2 mM dNTPs (Amersham Biosciences), 2.5 µl DMSO (SIGMA), 500 nM each of the forward and reverse primers, 2.5u PFU turbo polymerase (Stratagene). A control without genomic DNA was always run. The PCR programme used to amplify the fragments was 94°C for 2 min and 35 cycles of 94°C for 30s, 60-64°C for 30s, 68°C for 1min/kb. The 35 cycles were followed by a single additional stage of

68°C for 5 min. The extension time was changed according to fragment size, with 1 min per kilobase as a guideline. The annealing temperature was adjusted according to the predicted T_m of the primers as calculated by the DNASTAR primer design package. For generating some PCR products, PCR Supermix (Invitrogen) was used according to the manufacturer's instructions. 2.5u PFU turbo polymerase (Stratagene) was added in addition to the Taq polymerase already present. All PCR products were sequenced following cloning.

2.2.2 Restriction digestion

Restriction digests were set up with the appropriate amount of DNA, 1/10 total volume buffer, 1/100 volume BSA, 1/10 to 1/50 volume restriction enzyme, depending on activity and made up to the total volume using dH₂O. Restriction digests were incubated at the appropriate temperature for 1- 15 hours.

2.2.3 Agarose Gel Electrophoresis

DNA samples were run on a 0.8-1% agarose gel (Sigma) at 80V for 1 hour or 22V overnight. A BioDoc-it system (UVP) was used to visualise bands under UV light at 254 nm. Where bands were excised, the gels were visualised using a standard 3UV Transilluminator at 302 nm. The QIAquick Gel extraction kit (Qiagen) was used was used to extract DNA from agarose gels.

2.2.4 Preparation of DNA

Phenol-chloroform extraction using Phase Lock Gel tubes (Eppendorf), and ethanol precipitation was performed to purify the DNA prior to cloning. Equal volumes of restriction digest mix and buffered phenol:chloroform:isoamyl alcohol (Sigma) were added to a pre-centrifuged Phase Lock Gel tube and centrifuged for 2 min at 13,000 rpm (SIGMA 1-15K). The top fraction containing the DNA was removed and transferred to a new eppendorf tube with 2.5 X volume 96% ethanol and 1/10 volume 3M sodium acetate, pH6. This was incubated at -20°C for at least 1 hour and centrifuged at 4°C for 15 min at 13,000 rpm. The supernatant was removed and 100 µl 70% ethanol added, this was centrifuged for 5 min and all supernatant removed. The pellet was allowed to air dry at RT and resuspended in appropriate volume of 1X DNA dilution buffer (Roche) or dH₂O.

2.2.5 Methods for cloning

2.2.5.1 Standard cloning

Ligation was carried out using the Rapid DNA Ligation Kit (Roche). The insert and vector were quantified and approximately twice the amount of insert to vector was incubated with 1 X ligation buffer and 1u T4 DNA ligase, at RT for 10 min. Once ligation was completed, transformation with subcloning efficiency DH5α cells (Stratagene) was performed according to the manufacturer's instructions as follows: 10 µl ligation reaction was added to 50 µl DH5α cells and incubated on ice for 30 min. The reaction mix was heat-shocked at 37°C for 45 seconds, incubated on ice for a further 2 min and 950 µl L-broth added. This was then

incubated at 37°C for 1 hour with shaking at 250 rpm and various amounts plated out onto L-agar supplemented with the appropriate antibiotic.

2.2.5.2 Cloning using the TOPO vector

Selected PCR products, where standard cloning was inappropriate, were cloned blunt into a TOPO vector, pCR-4 blunt (Invitrogen). The TOPO® Cloning Reaction kit (Invitrogen) was used and transformation was via the One Shot® Mach1™-T1® Chemically competent cells (Invitrogen). The cloning reaction was set up as follows: 0.5-4 µl PCR product was added to 1µl salt solution. Sterile water was added to a total volume of 5 µl and 1 µl TOPO vector added. The reaction was mixed gently and incubated for 5 min at RT. For the transformation 2 µl of the cloning reaction was added to a vial of One Shot chemically competent *E. coli* and incubated for 5 min on ice. The cells were heat-shocked for 30 seconds at 42°C without shaking and transferred to ice. 250 µl of RT SOC medium was added and the tube shaken horizontally (250 rpm) for 1 hour at 37°C. 10-50µl from each transformation was plated onto a pre-warmed L-agar plate supplemented with kanamycin or ampicillin. Plates were incubated overnight at 37°C.

DNA was extracted from the clones obtained using a QIAprep kit (Qiagen) and digested with the appropriate restriction enzyme. The desired fragment was excised from an agarose gel and was then cloned conventionally into the appropriate vector.

2.2.6 Mutant plasmid construction

In order to make the targeting constructs (Table 2.2), 5' and 3' regions flanking the gene of interest were amplified using PCR as described previously. These were then cloned into pBackbone (Gopaul 2003), a suicide vector containing kanamycin and ampicillin resistance cassettes. A gentamycin resistance marker was inserted between the 5' and 3' fragments of the gene to ensure loss of function of the gene and to facilitate selection of mutants. The *sacB/lacZ* cassette, cut out from pGoal-17 and gel purified, was inserted into the *PacI* site of pBackbone. This cassette enabled blue/white screening on X-gal enriched media and counterselection against the vector in the final knockout selection stages in *M. tuberculosis*.

Table 2.2: Targeting constructs

The targeting constructs made to mutate the genes under study.

Plasmid	Construct	5' fragment (bp)	3' fragment (bp)
pRC3	pBackbone targeting Rv0937c, with Gm ^R cassette and SacB/LacZ cassette	1644	1760
pRC4	pBackbone targeting Rv0938, with Gm ^R cassette and SacB/LacZ cassette	2056	1941
pRC5	pBackbone targeting Rv0937c and Rv0938, with Gm ^R cassette and SacB/LacZ cassette	1644	1941
pRC7	pBackbone targeting Rv2191, with Gm ^R cassette and SacB/LacZ cassette	1174	1227
pRC11	pBackbone targeting Rv3395c, with Gm ^R cassette and SacB/LacZ cassette	2236	2244

2.2.7 Extraction of plasmid DNA

DNA was extracted from 5ml cultures grown up overnight with shaking, using the QIAprep kit (Qiagen) according to the manufacturer's instructions. For screening large numbers of plasmids, DNA was extracted from 1ml culture grown overnight with shaking, using STET preps (Holmes and Quigley 1981). Plasmid identity at each step was checked using multiple restriction digests and when confirmed, completed knockout constructs were electroporated into H37Rv.

2.2.8 Electroporation and counter selection in *M. tuberculosis*

Competent cells were prepared by growing H37Rv to an OD₆₀₀ of 1.0 and adding 1/10 volume 2M glycine. Cells were grown for a further 24h, harvested and then washed 4 times in 10% glycerol (10,000 rpm, Sorvall RC-5B). Cells were resuspended in 1/10th volume glycerol and stored at 4°C.

1-5µg of plasmid DNA was electroporated into 400µl H37Rv competent cells at 2.5kV, 25 µF, 1000Ω (Biorad). A control containing no DNA was also electroporated. The cells were incubated statically overnight at 37°C and then concentrated by centrifugation (10,000 rpm, Jouan BR4) and plated onto 7H11 plates containing Gm (15 µg/ml) and X-gal (100 µg/ml). Plates were incubated for 4 weeks at 37°C. Any white colonies were potential double crossovers and these were picked and patched onto 7H11+Gm, and 7H11+Km to look for gentamycin resistant and kanamycin sensitive colonies, from which DNA was extracted. Blue colonies were picked and streaked onto 7H11+Gm plates and incubated at 37°C for 3-4 weeks to allow a second crossover to occur. Colonies were picked and

serially diluted in modified Dubos + 4% albumin and dilutions plated onto 7H11+Gm+Xgal+2% sucrose. This was the counter-selection step against the *sacB* gene, present in the pBackbone vector, which confers sucrose sensitivity. Sucrose resistance enriches for loss of the original vector. The dilutions were incubated at 37°C for 4 weeks and the white sucrose-resistant colonies which were potential double crossovers were picked and patched onto 7H11+Gm and 7H11+Km. Blue colonies present at this stage are spontaneous *sacB* mutants. White colonies that were gentamycin resistant and kanamycin sensitive were streaked onto 7H11+Gm plates for DNA extraction. This process is shown schematically in figure 2.1

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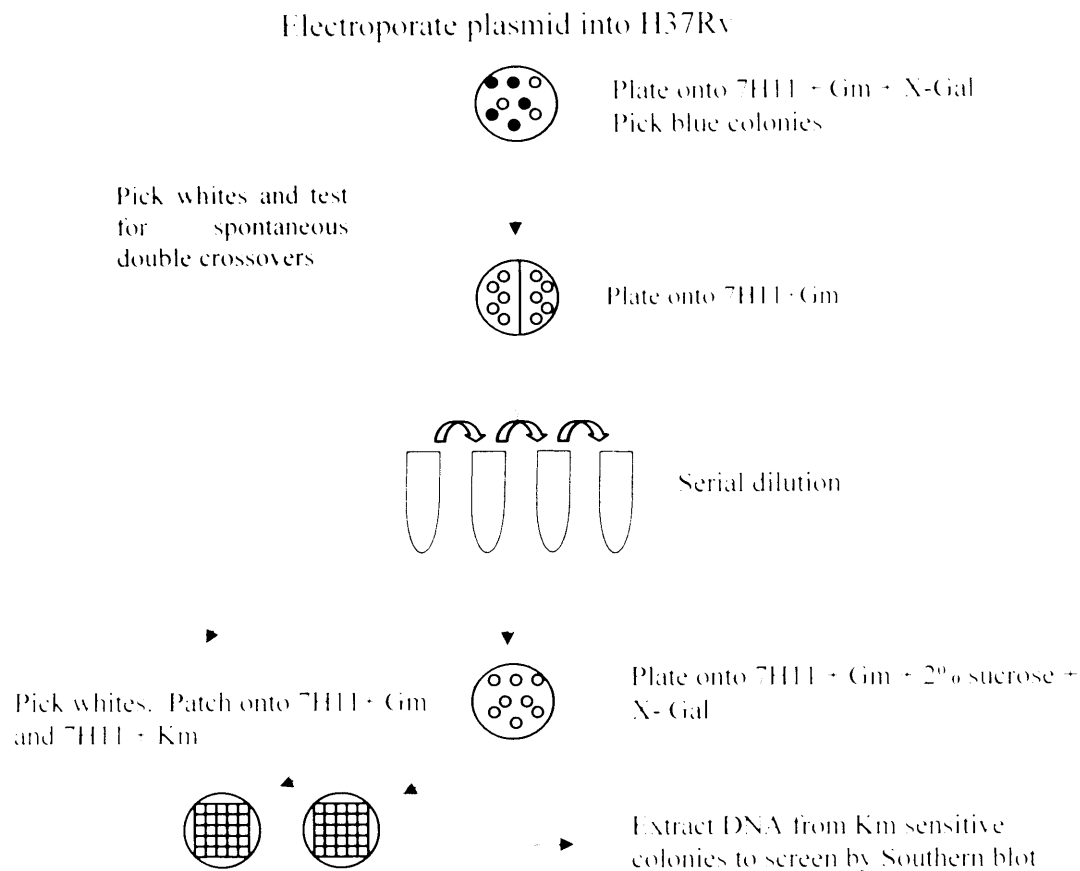


Figure 2.1: Electroporation and counterselection in Mtb

The figure shows a schematic version of selection stages leading to identification of potential double crossovers. Plasmids are electroporated into H37Rv and plated out to select for the first crossover event. After the step to allow the second crossover event, colonies are serially diluted and streaked onto plates containing 2% sucrose, for counterselection, and X-gal. Potential double crossovers are tested for Km sensitivity in the final stage and then screened by Southern blot. Occasionally it is possible to obtain a spontaneous double crossover at the initial selection stage. The entire process takes around 6 months.

2.2.9 DNA extraction from *M. tuberculosis*

Cells were harvested from a quarter of a plate, resuspended in 400 μ l TE buffer and incubated at 80°C for 1h. 20 μ l lysozyme and lipase solution in TE buffer, to give a final concentration of 2 mg/ml each, were added to the resuspended cells with 1 μ l RNase, 500 μ g/ml (DNase-free RNase, Roche). The tubes were incubated at 37°C for 2h and frozen at -20°C for 30min. Tubes were thawed at 50°C for 10 min and proteinase K (Boehringer, final conc. 500 μ g/ml), SDS (0.5% final conc.) and 5 μ l RNase (500 μ g/ml) were added. Tubes were incubated at 50°C for 1h and extracted with phenol:chloroform:isoamyl alcohol (SIGMA). Supernatants were removed and transferred to Phase Lock Gel tubes (Eppendorf) and extracted with an equal volume of chloroform. Each supernatant was removed and 1/50th volume 5M NaCl and 2 volumes absolute ethanol added. The tube was gently inverted and the chromosomal DNA precipitated like a thread. This was centrifuged at 13,000 rpm for 15 min (SIGMA 1-15K) and washed with 70% ethanol, re-centrifuged and the pellet air dried. The DNA was dissolved in 300 μ l TE buffer and incubated overnight at 37°C before storing at 4°C.

2.2.10 Southern Blotting

Genomic DNA (3 μ g) was digested overnight with the restriction enzymes selected and loaded on a 0.8% agarose gel (SIGMA). Samples were run overnight at 22V and the gel photographed using a gel imager (BioDoc-it). The gel was incubated in 0.25M HCl for 15min, rinsed with dH₂O and placed in denaturation buffer (1.5M NaCl, 0.5M NaOH) for 30 min. The gel was rinsed in dH₂O and incubated with neutralisation TNE buffer (1.5M NaCl, 0.5M Tris pH 7.2, 0.001M EDTA),

rinsed in dH₂O and blotted overnight in 20 X SSC (Maniatis *et al.*, 1982). The blot was set up in a container with SSC, and a piece of Whatman paper acting as a wick. The gel and a Hybond-N⁺ membrane (Amersham Biosciences) cut to the size of the gel were laid over this. 3 further pieces of Whatman paper were laid on the membrane with a pack of tissues over them held down by a glass plate and 2 X 1kg weights on the top. The membrane was washed for 5 min in 2 X SSC, air dried and UV treated, (optimal crosslink, Spectrolinker: 1200 X 100µJ/cm²).

2.2.10.1 Radioactive hybridisation

The blot and gel sized piece of Nitropore mesh, to aid rolling of the membrane, were incubated in a Hybaid tube with 2 X SSC containing salmon sperm DNA (10 mg/ml), 5% X SSC, 5 X Denharts solution and 0.5% SDS at 65°C for 2h.

The purified probe was denatured at 100°C for 5 min and the purified probe solution (purified probe + dH₂O) was added to dry Ready-to-go labeling beads (Amersham Pharmacia Biotech) and 4 µl α³²P dCTP added to label the probe. The solution was incubated at 37°C for 30 min and passed through a Sephadex column (Amersham) to remove unincorporated label. The labeled probe was added to the blot and incubated at 65°C overnight. The membrane was washed twice with 2 X SSC + 0.1% SDS for 15 min at RT, 0.1 X SSC + 0.1% SDS once for 15 min at RT, once for 15 min at 55°C and twice for 15 min at 65°C. The membrane was then taped to a cassette and exposed to a film for various time intervals.

2.2.10.2 Non-radioactive hybridisation

ECL direct nucleic acid labelling and detection system (non-radioactive Southern) kit was used (Amersham Biosciences). The UV cross-linked blot was immersed in 5 X SSC, loosely rolled with a nitropore nylon membrane and unrolled in a Hybaid tube containing 20ml 5 X SSC. The SSC was poured off and 25 ml hybridisation buffer added (0.5M NaCl containing 5g blocking agent in 100ml hybridisation buffer, Amersham, both provided with kit). The blot was pre-hybridised in a hybaid oven at 42°C for 45 min. Probe DNA was diluted to a concentration of 10ng/μl. The ladder probe (Lambda /*Hind*III + *Eco*RI) was also diluted in this way. 100ng of both probes (100μl) were denatured by heating for 5 min at 100°C and then immediately cooled on ice for 5 min. The equivalent volume of DNA labelling reagent (Amersham Biosciences, provided in kit) was added to cooled DNA, mixed and the same volume of gluteraldehyde (Amersham Biosciences, provided in kit) added. The samples were incubated at 37°C for 10 min and added to the pre-hybridisation buffer. The blot was then hybridised overnight at 42°C. The blot was washed once with 5 X SSC for 5 min, and three times with primary wash buffer (6M urea, 0.4% SDS, 0.5 X SSC) pre-warmed to 42°C for 10-20 min per wash. The blot was removed from the hybridisation tube and incubated in 2 X SSC for 5 min with shaking. Signal generation and detection was carried out according to ECL Direct Nucleic Acid Labelling System, Detection Reagents (Amersham Biosciences) protocol. The membrane was incubated with detection reagents for 1 min, taped to a cassette and exposed to Hyperfilm (Amersham Biosciences) for various periods of time.

2.2.11 Complementation

A complementing strain was constructed in order to re-insert the gene back into the mutant. Primers were designed in the flanking region on each side of the gene (see Appendix II for sequence) and the product was generated using PCR as described previously. Once the PCR product was obtained, it was cloned blunt into a TOPO vector, pCR-4 blunt (Invitrogen), as described previously. The cloned fragment was sequenced within the TOPO vector and extracted by digesting the plasmid with *Xba*I. The fragment was then cloned into the *Xba*I site of pKP186 (Rickman *et al.*, 2005).

To confirm that the fragment was in the plasmid, a series of restriction digests were carried out. 1 µg plasmid and 300ng pBS-integrase were electroporated into ΔRv2191 competent cells made using the standard protocol as described for wild type H37Rv. The addition of the *integrase* gene carried on a separate vector maintains stability of the re-inserted gene and prevents spontaneous excision associated with using an integrated vector (Springer *et al.*, 2001).

Electroporations were inoculated into 5ml Dubos + albumin and incubated overnight at 37°C before plating onto 7H11+Km plates. Colonies were picked and restreaked after 4 weeks and allowed to grow for a further 4 weeks prior to a single colony being picked, streaked, and inoculated into 5ml Dubos + albumin to grow for 1 week prior to inoculation into liquid culture.

2.3 Phenotypic characterisation of *M. tuberculosis* mutants

2.3.1 Growth *in vitro*

Ability of mutants to grow *in vitro* was measured alongside the wild type strain as a comparison. A sample of culture from a 5 ml standing culture was added to 50 ml Dubos media, according to a calculation to obtain an OD₆₀₀ as close to 0.01 as possible. Cultures were incubated at 37°C with rolling for a period of 12 days. Growth was measured as the increase in optical density during this period and recorded every 24 h.

2.3.2 Exposure to DNA damaging agents using filter discs

Two cultures of each strain were grown to an OD₆₀₀ of 0.3-0.4. 50 µl was aliquotted onto 7H11 + OADC plates and spread using a sterile cotton swab (Fisher). Once the culture was absorbed into the plate, 3 X 6 mm filter discs (Oxoid) were put on each plate, equidistant from each other. Discs were prepared by adding 10 µl of selected concentrations of DNA damaging agents and allowed to dry. Standardisation was performed using varying concentrations of DNA damaging agents to determine the optimal concentration for the experiment. DNA damaging agents used were bleomycin (7.5 µg per disc), mitomycin C (20 µg per disc), ofloxacin (4 µg per disc) and menadione (70 µg per disc).

2.3.3 Exposure to gamma radiation

Exposure to gamma radiation was investigated during exponential phase. Cultures of each strain were grown to an OD₆₀₀ of 0.3-0.4. In order to comply with safety

regulations, 40ml aliquots for each strain were held within a sealed Falcon tube inside a plastic centrifuge tube. Samples were subjected to 50 or 100 Greys from a Caesium source. A control sample was left untreated. Aliquots were returned to rolling (2 rpm at 37°C) for 24 hours, and 50 µl serially diluted in 450µl 50% DMEM and fetal calf serum. 10 µl was streaked onto quarter of a plate (7H11 + albumin) using a plastic loop. Plates were incubated at 37°C for 14 days. Colonies were then counted.

Additionally, selected mutant strains were grown to an OD₆₀₀ of at least 2.0 and exposure to gamma radiation measured in stationary phase. 40 ml aliquots for each strain were then subjected to gamma radiation as described above or left untreated as a control. Aliquots were returned to rolling at 37°C for 24 hours prior to serial dilution and plating.

2.3.4 Viability assays

Viability assays were carried out based on a protocol kindly provided by Dr Lucinda Rand, (Novartis, Singapore). Mtb strains were grown to an OD₆₀₀ of 0.3-0.4. 190 µl aliquots were added to 10 µl DNA damaging compound in a 96 well plate. The lid was taped on and the plate placed inside a plastic sandwich box. Boxes were incubated at 37°C for 24h or 6d. After the designated incubation step, samples from each well were serially diluted with sterile saline, using a multi-channel pipette. A 25 µl sample was plated out from the required dilutions. Plates were allowed to dry and incubated at 37°C or 15d. The experiment was standardised specifically for each individual compound used. DNA damaging

agents used were menadione (100 μ M), t-butyl hydroperoxide (250 μ M), hydrogen peroxide (2 mM), diamide (10 mM) and acidified sodium nitrite (3 mM).

2.3.5 Repair efficiency of mutants

A replicating plasmid pMV261 (Stover *et al.*, 1991) was digested with one of three enzymes; *Pvu*II (blunt), *Pst*I (3' overhang) or *Eco*RI (5' overhang), incubated overnight and gel extracted. Linearised DNA was quantified using a Nanodrop ND-1000 Spectrophotometer and 50 ng cut DNA or 50 ng uncut circular DNA as a control was electroporated into 400 μ l Mtb competent cells. Wild type, Δ Rv0937c, Δ Rv0938 and Δ Rv0937c/Rv0938 competent cells were used for electroporation. After electroporating, samples were incubated overnight at 37°C in 5 ml Dubos media and for samples transformed with cut DNA, 100 μ l was plated out. For samples transformed with uncut DNA, 100 μ l of a 1 in 20 dilution was plated out. Colonies were counted after 3-4 weeks growth and transformation efficiency calculated. As a control to assess the efficiency of linearisation, the cut and uncut plasmids were transformed into *E. coli*, 50 ng of cut DNA and 1 and 10 ng uncut DNA were transformed. Various amounts were plated out and incubated overnight. Colonies were then counted. (Protocol was adapted from Gong *et al.*, 2005)

A control experiment was performed to assess the rate of random integration. This was carried out by electroporating a non-integrating plasmid, pBackbone (Gopaul 2003) into the competent cells as a control. The plasmid was cut using restriction

enzymes to give blunt, 3' and 5' ends prior to electroporation. The number of colonies obtained per electroporation were counted and compared between strains.

2.4 *In vivo* characterisation of *M. tuberculosis* mutants

2.4.1 Survival in macrophages

Bone marrow cells were extracted from the hind legs of female BALB/C mice (Tascon *et al.*, 2000), provided by Dr Edward McGowan. The extraction was carried out with the assistance of Mr Steven Coade. The cells were resuspended in Iscove's modified Dulbecco's medium supplemented with 5% fetal calf serum, 2mM L-glutamine and 80mM β -mercaptoethanol. 10% by volume of supernatant from L929 cells that produce macrophage colony-stimulating factor was added to the β -mercaptoethanol (Curry *et al.*, 2005). The cells were plated into 12 well plates and incubated at 37°C. After 24 h, the cells were washed with fresh medium and non-adherent dendritic cells removed. Fresh medium was added to the macrophages and the plates were incubated for 3 days at 37°C. After 5 days' growth, cells were infected with Mtb (day 0). Bacteria were added at a multiplicity of infection of 1 bacteria per 2 macrophages. 50,000 bacteria were added to 100,000 macrophages. A sample was taken for serial dilution and plating, to determine viability. For the activated macrophages, bacteria were added from frozen stocks, where viability had been previously determined by freezing samples and plating out serial dilutions. This allowed a more precise estimate of bacterial numbers.

Following infection, the macrophages and bacteria were incubated for 5 hours, after which time, the medium was removed and replaced with fresh medium. At each time point, 2% Saponin was added to the relevant wells and the samples incubated for 1 h, to lyse the macrophages. Samples were serially diluted and plated out onto Middlebrook 7H11 plates supplemented with 4% Dubos medium albumin. This was carried out on days 0, 1, 3, 6 and 10 for growth in unactivated macrophages and days 0, 2, 5 and 8 for activated macrophages. Macrophage viability was checked using a microscope at each timepoint.

For activated macrophages, recombinant mouse IFN- γ (R & D systems) was used at a final concentration of 100u/ml and bacterial *E.coli* LPS (Roche) at a concentration of 1 μ g/ml. These were added to the macrophages 24 hours prior to the addition of the bacteria and again after washing on day 0. Activation level of the macrophages was checked by measuring the level of nitric oxide, using a Parameter Total NO/Nitrite/Nitrate assay kit (R & D Systems), as per manufacturer's instructions

2.4.2 Survival in mice

Mouse model experiments were carried out by Mr John Brennan. These were carried out in the Category 3 containment animal house. Stock cultures of wild type and mutant Mtb strains were grown in 10 ml Dubos medium standing cultures at 37°C for 2 weeks. The cultures were diluted in phosphate-buffered saline to an OD₆₀₀ of 0.02. Female BALB/c mice were infected via injection into the lateral tail vein with 0.2 ml Mtb. A viable count was carried out to determine bacterial viability. At specific time points, the lungs and spleen were harvested,

homogenised and serially diluted onto Middlebrook 7H11 plates, supplemented with Dubos medium albumin. Plates were incubated and colonies counted after growth for 15 days.

3 Targeted mutation in *M. tuberculosis*

Mutant strains of *M. tuberculosis* (Mtb) were constructed, in which the selected gene was inactivated by deletion. The genes were chosen because they were either DNA damage inducible or thought to be involved in DNA damage repair.

3.1 Introduction

Construction of a knockout strain enables investigation into the phenotype of the mutant and examination of the potential role of the gene in survival under various DNA damaging conditions. It is also possible to investigate whether the mutated gene plays a role in pathogenesis and/ or growth. Constructing mutants in Mtb is a lengthy and technically challenging operation, with numerous stages both in the construction of the targeting construct, and following electroporation into Mtb. The process can be expected to take a minimum of 6 months due to the slow growth of the organism, the technical challenges imposed by the cloning and selection process itself, and constraints imposed by working within category 3 containment.

The genes selected for mutation studies were Rv0937c and Rv0938, components of the non-homologous end joining pathway, Rv2191, predicted to be involved in nucleotide excision repair, and Rv3395c, a DNA damage inducible gene. A double mutant, where both Rv0937c and Rv0938 were deleted, was also constructed. This will allow further characterisation of the role of NHEJ as the entire pathway is inactivated in the double mutant.

To ensure that the gene function is inactivated, it is important to ensure that the deleted region encompasses a large proportion of the functional domains of the encoded protein. This was confirmed by checking the locations of predicted domains using the NCBI conserved domain database (<http://www.ncbi.nlm.nih.gov/Structure>) and designing primers accordingly (Figures 3.1-3.4). There is no useful domain prediction for Rv3395c as yet.

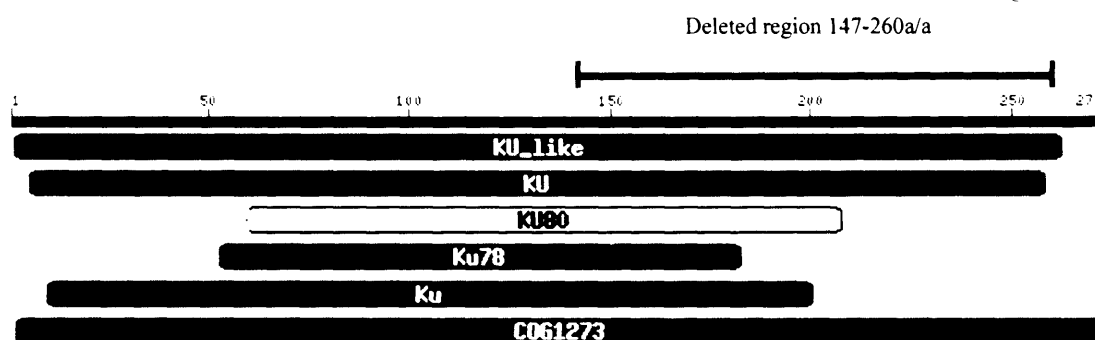


Figure 3.1: Domain prediction for Rv0937c from the NCBI Conserved domain search

(<http://www.ncbi.nlm.nih.gov/Structure>)

The region with highest score is a Ku-like domain (100% alignment), which includes the core domain present in prokaryotic Yko-V-like proteins and eukaryotic Ku70 and Ku80 domains. The deleted region in the mutant (147-260 amino acids) is indicated by the green line.

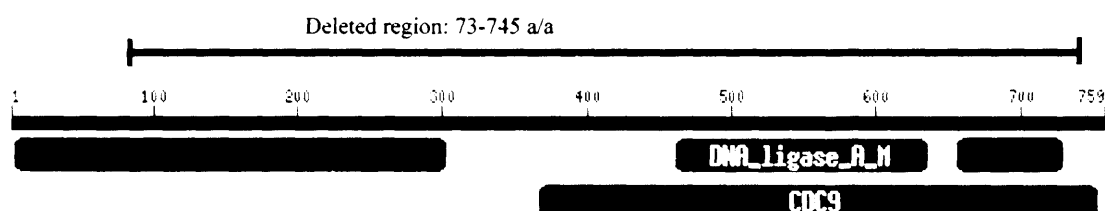


Figure 3.2: Domain prediction for Rv0938 from the NCBI Conserved domain search

(<http://www.ncbi.nlm.nih.gov/Structure>)

The regions with highest score are the ATP dependent DNA ligase domain, an ATP dependent DNA ligase C terminal region, which is found in most ATP-dependent DNA ligase enzymes and is thought to constitute part of the catalytic core of ATP dependent DNA ligase. There is also a predicted eukaryotic-type DNA primase domain. The deleted region in the mutant (73-745 amino acids) is indicated by the green line, and removes nearly the entire functional domains.

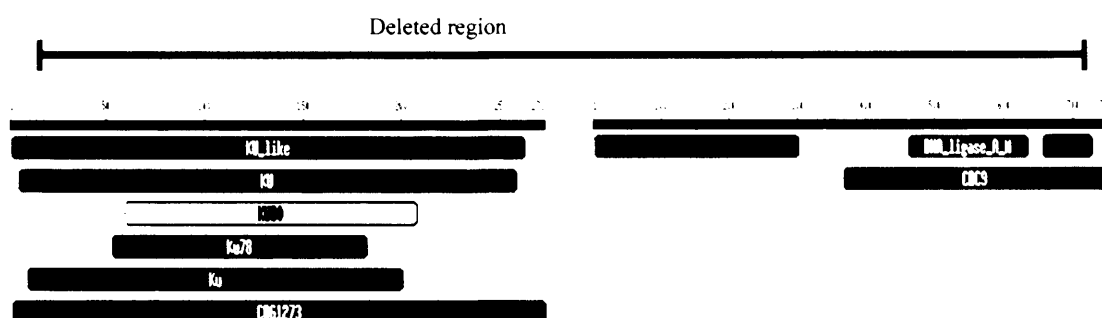


Figure 3.3: Domain prediction for Rv0937c and Rv9038 double mutant from the NCBI Conserved domain search

(<http://www.ncbi.nlm.nih.gov/Structure>)

The green line indicates the deleted region in the mutant. As the genes are divergently transcribed, the deletion results in removal of essentially the entirety of both genes. Almost the entire functional domains for Rv0937c and Rv0938 are disrupted in this way.

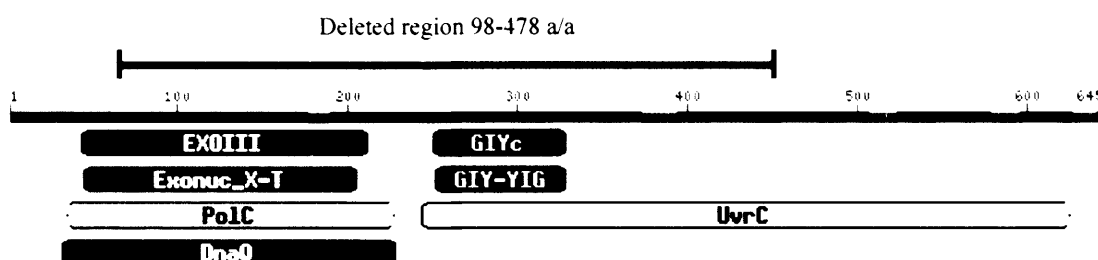


Figure 3.4: Domain prediction for Rv2191 from the NCBI Conserved domain search

(<http://www.ncbi.nlm.nih.gov/Structure>)

Regions with highest score are shown on the top line. These include an EXOIII exonuclease domain and a GIYc, GIY-YIG type nuclease. The deleted region in the mutant (98-478 amino acids) is indicated by the green line.

3.2 Constructing knockouts

A suicide vector was used to construct the knockouts. The plasmid used was pBackbone (Gopaul 2003), which contains a kanamycin resistance cassette. Following insertion of the target sequence, a *sacB/lacZ* cassette was cloned into the *PacI* site to aid in the selection stages after electroporation. The utilisation of a suicide vector such as this means that resistance to the antibiotic it carries should be dependent on integration into the chromosome and therefore depends on homologous recombination events between the sequences cloned in the plasmid and the sequences flanking the region to be deleted in the bacterial genome (Hinds *et al.*, 1999).

Primers were designed in the flanking regions of the 5' and 3' ends of the gene to be knocked out, in order to generate two PCR products predominantly in the regions flanking the gene (Figure 3.5). The mutated gene would then have impaired function when introduced back into the host as it would be missing a large portion. Restriction sites were added at one end of each pair for directional cloning into the vector. 3' fragments were cloned into the *EcoRI*-*EcoRV* sites of pBackbone and the 5' fragment were cloned into the *Ecl136II*-*XbaI* sites.

In addition, a gentamycin resistance cassette was cloned into the *XbaI* site, between the two fragments, to further disrupt any remaining gene function and facilitate selection of mutants. Finally, a *sacB/lacZ* cassette was added at the *PacI* site. The *lacZ* gene codes for β -galactosidase which turns colonies blue in the presence of X-gal and expression of *sacB* confers sensitivity to sucrose and is used as a negative selectable marker against single crossovers in Mtb (Pelicic *et*

al., 1996). In each case, the identity of the plasmid was checked using multiple restriction digests and the targeting construct was electroporated into H37Rv. Following electroporation, the ensuing transformants underwent a series of selections as described below, and finally double crossovers were identified by Southern blot.

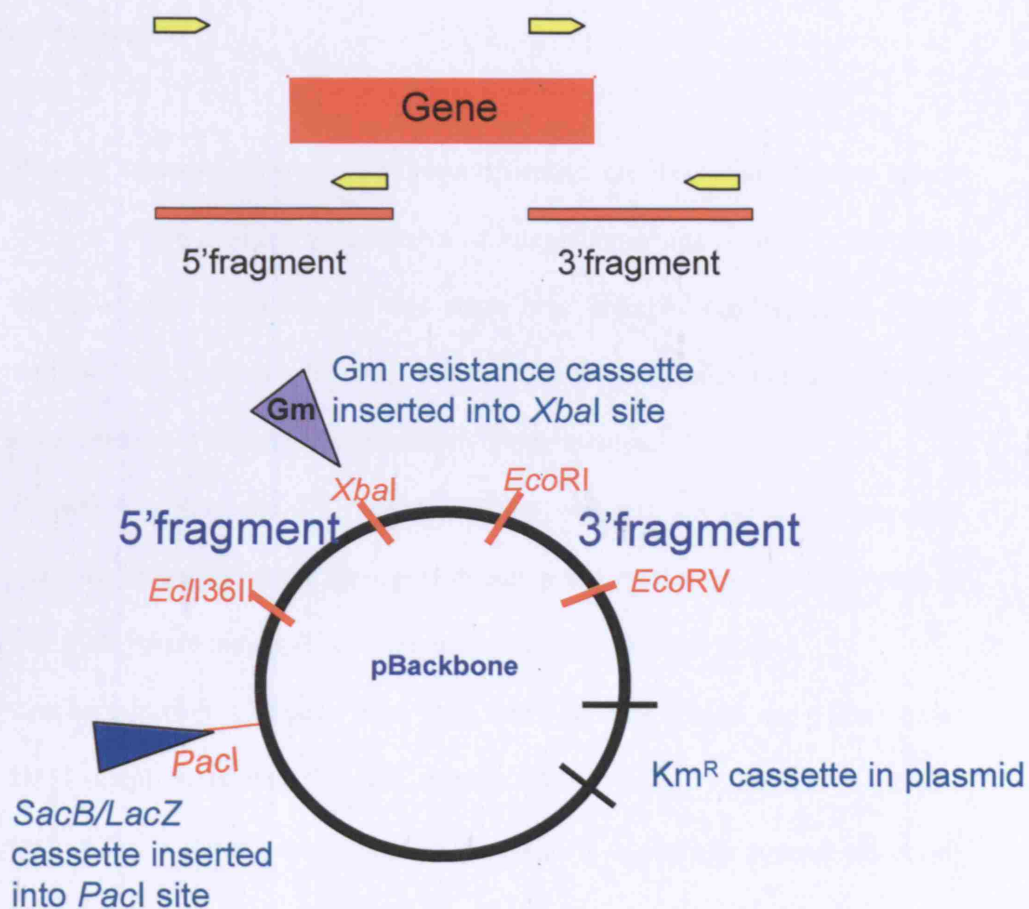


Figure 3.5: Making knockout constructs in *E. coli* for electroporation into Mtb

3' and 5' fragments were generated by PCR and then directionally cloned into the pBackbone vector. The 3' fragment was inserted between *EcoRI* and *EcoRV*, the 5' fragment was inserted between *XbaI* and *Ecl136II*. A Gm^R cassette was inserted between the 2 fragments, into the conserved *XbaI* site and finally a *sacB/lacZ* cassette was inserted into the *PacI* site. The antibiotic resistance markers and *sacB/lacZ* cassette were used in the various selection stages to confirm loss of the original plasmid.

The selection procedure applied to the Mtb transformants can be divided into the following stages:

- 1) Primary selection: Growth after electroporation on 7H11+Gm+Xgal to select for Gm^R (4-6w), which is indicative of integration of the plasmid or isolation of the desired mutation. At this stage blue colonies can represent single crossovers or random integrants. White colonies can arise as a result of double crossovers or spontaneous mutation to Gm resistance.
- 2) Growth to allow the 2nd crossover: Blue colonies (single crossovers and random integrants) from the electroporation are picked and streaked onto a Gm plate where they will be white in the absence of X-gal (3-4w).
- 3) Counterselection: Colonies from step 2 are serially diluted and plated onto 7H11 plates containing Gm, 2% sucrose (Pelicic *et al.*, 1996) and X-gal (3-4w). White colonies are picked. This stage is a sucrose counter-selection against the vector via *sacB*. X-gal is added to the plates to aid the screen as it is possible to obtain spontaneous *sacB* mutants. White colonies on X-gal enriched media indicate absence of this *lacZ*, and hence loss of the original vector.
- 4) Confirmatory phenotypic screen: White colonies from both steps 1 and 3 are picked and patched onto separate plates containing either Km or Gm. The desired phenotype is Gm^R and Km^S to confirm loss of the original vector (3-4w).
- 5) Verification of the desired phenotype by Southern blot. DNA is isolated from colonies with the correct phenotype. The genetic arrangement at the targeted locus is assessed using one or more labelled probes in this region.

Table 3.1: Gene knockouts under investigation

This table shows the targeting constructs used to make mutants in Mtb.
Primer sequences can be found in appendix II.

Construct	Gene targeted	homology	Gene Size	Deleted region	Function
pRC3	Rv0937c	Ku	822bp	343bp 439-781	Involved in NHEJ pathway of DNA repair
pRC4	Rv0938	Ligase	2280bp	2021bp 217-2237	Involved in NHEJ pathway of DNA repair
pRC5	Rv0937c/ Rv0938	Ku+Ligase	822+2280 + intergenic region	3154bp 37-3191*	Involved in NHEJ pathway of DNA repair
pRC7	Rv2191	Cho	1938bp	1143bp 292-1434	Involved in NER pathway of DNA repair
pRC11	Rv3395c	-	615bp	243bp 190-432	Unknown function

* The deleted region coordinates for pRC5, the double Rv0937c/ Rv0938 mutant, represent an area deleting regions of Rv0937c and Rv0938 together.

3.2.1 Recombination events in *M. tuberculosis*

In order for the desired mutation to be successfully introduced into the targeted gene resulting in its inactivation, recombination events must occur in each of the 3' and 5' flanking regions during the selection processes. In this way, the plasmid DNA is removed and the mutated gene inserted into the bacterial DNA, replacing the wild type sequence, (Figure 3.6). Different recombination events can be distinguished when screened by Southern blot as they will result in different sized bands following digestion with appropriate restriction enzymes, when using a probe that binds within the flanking regions.

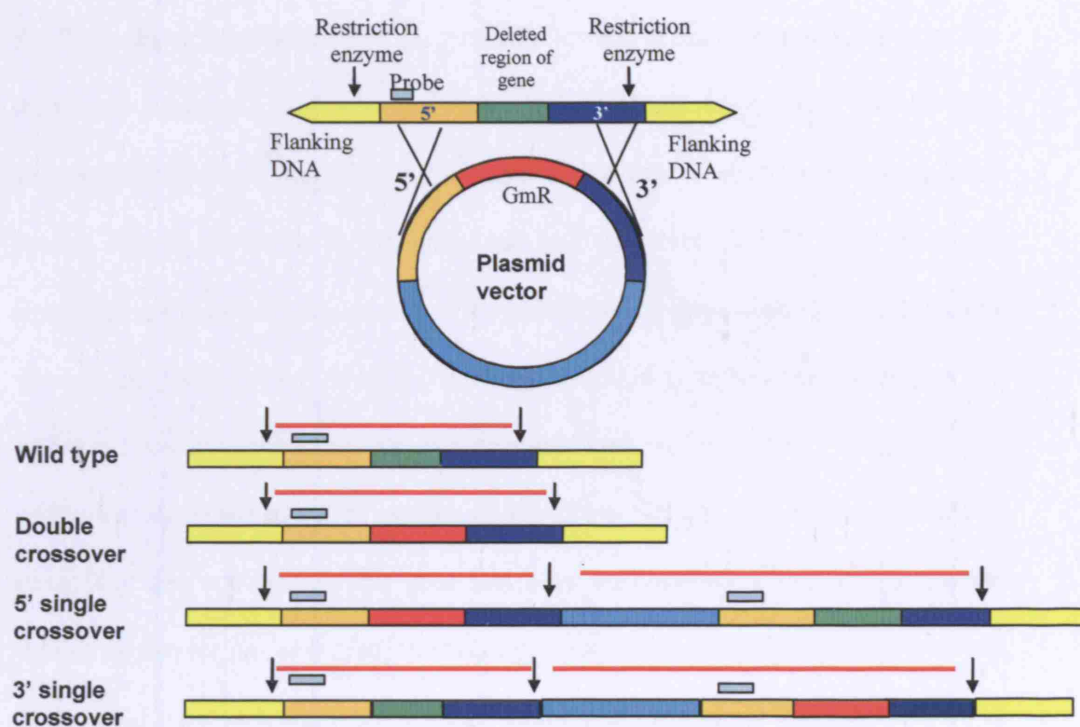


Figure 3.6: Recombination events in *M. tuberculosis*

Schematic illustration of the possible recombination events for generating mutant strains in Mtb. Arrows indicate cutting sites of the restriction enzymes that lead to different sized fragments, which can be identified by Southern blotting. Small blue boxes represent the location of probe binding. The number of binding sites depends on the recombination event that has occurred.

3.2.2 Screening of potential knockouts by Southern Blot

Southern blots were performed on potential double crossovers resulting from the series of selections and arising from the initial electroporation which were gentamycin resistant, kanamycin sensitive and white. Restriction enzymes were chosen which cut twice in the knockout and wild type, ideally once in the 5' flanking region of the gene and once in, or near to the gene itself (Figure 3.7). The size of the fragment would differ between the wild type and the mutant, as the knockout has an antibiotic resistance cassette between the 5' and 3' fragments. A probe was designed in the 5' region, which allows insight into the recombination event that has occurred. If the gene has been successfully knocked out and the double crossover has occurred, the band present on the blot differs in size to that of the wild type, as long as the inserted gentamycin resistance fragment differs in size from the deleted region. Single crossovers can occur where only one side of the flanking region has undergone homologous recombination and these can be distinguished by the presence of two hybridising bands.

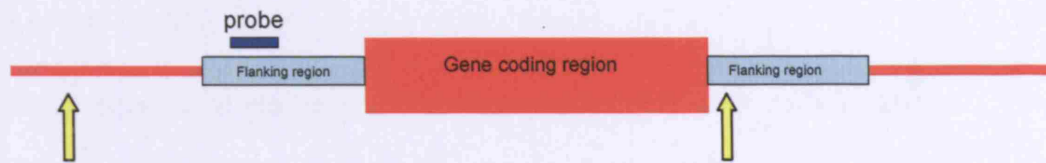
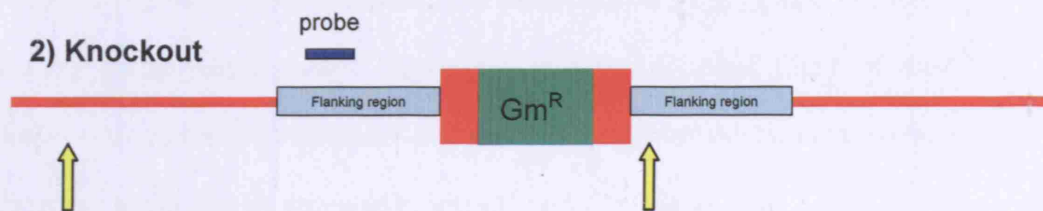
1) Wild type**2) Knockout**

Figure 3.7: Probe location for Southern blots

The probe is indicated by the dark blue box. Yellow arrows indicate the positions of cuts made by restriction enzymes. The size of the hybridizing band should vary between mutant and wild type.

3.3 Mutant strains constructed

3.3.1 Δ Rv0937c

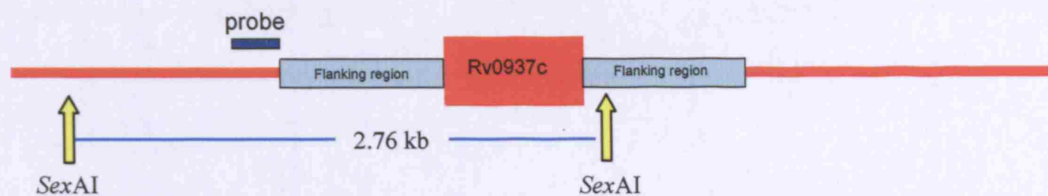
After an initial failed electroporation, which yielded only white colonies that all turned out to be single crossovers, two blue colonies were obtained from the subsequent successful electroporation. The serial dilution stage yielded 17 sucrose resistant, gentamycin resistant, kanamycin sensitive colonies. Eight of these colonies were screened by Southern blot and 5 of these were double crossovers. A radioactive Southern blot was used to identify mutants (Figure 3.8).

3.3.2 Δ Rv0938

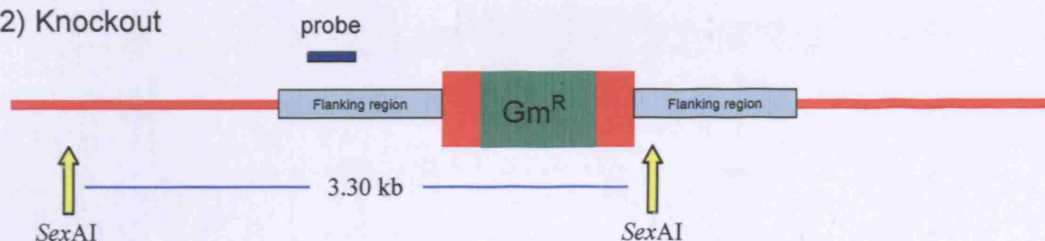
Four blue colonies were obtained from the second electroporation and 32 white colonies arose from the sucrose counterselection stage. Four of these were screened by a non-radioactive Southern blot and all were subsequently confirmed to be double crossovers (Figure 3.9).

a) Location of the probe

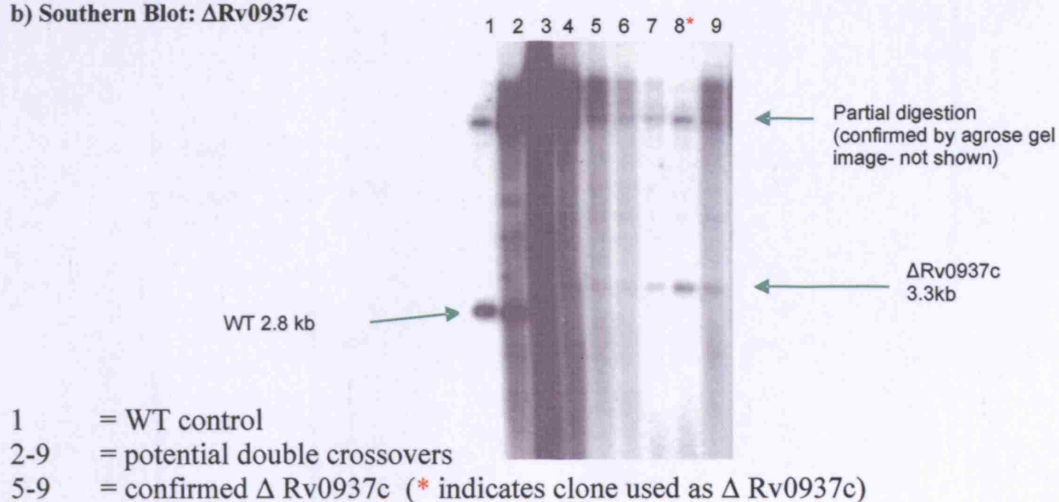
1) Wild type



2) Knockout



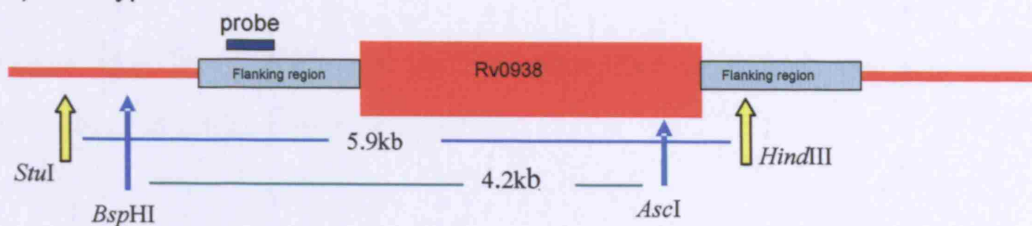
SCO bands?

b) Southern Blot: $\Delta Rv0937c$ **Figure 3.8: Southern blot and probe location for Rv0937c mutant**

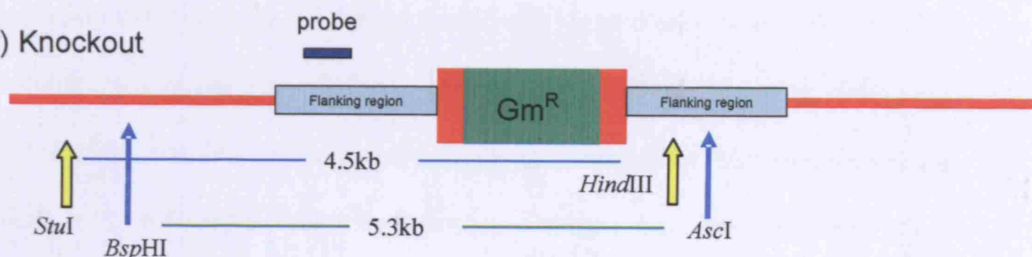
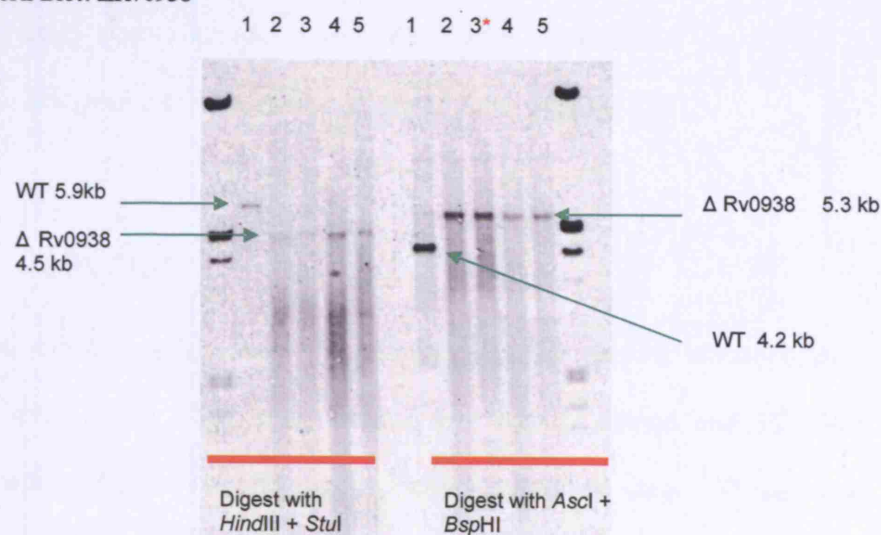
The figure shows the location of the probe with the restriction sites used to cut out the fragment used for screening double crossovers (a). Due to the insertion of the Gm^R cassette between the flanking regions of the gene, the WT and mutant will give different sized bands which can be distinguished by Southern blot (b). 3 μ g WT and 3 μ g potential double crossover DNA were digested with *SexAI*.

a) Location of the probe

1) Wild type



2) Knockout

b) Southern Blot: Δ Rv0938

1 = WT

2-5 = Δ Rv0938(*used as Δ Rv0938)**Figure 3.9: Southern blot and location of probe for Rv0938 mutant**

The figure shows the location of the probe with the restriction sites used to cut out the fragment used for screening double crossovers (a). Due to the insertion of the Gm^R cassette between the flanking regions of the gene, the WT and mutant will give different sized bands which can be distinguished by Southern blot (b). Two separate digests were used to confirm double crossovers. 3 μ g WT and 3 μ g potential double crossover DNA were digested with either *Hind*I and *Stu*I, or *Bsp*HI and *Asc*I

Mutating both the Rv0937c and Rv0938 genes should eliminate the non-homologous end joining system of repair.

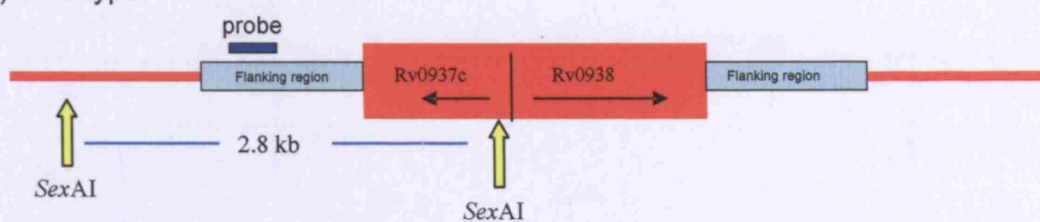
The initial electroporation was unsuccessful and yielded only white colonies. The subsequent electroporation yielded only one blue colony. However, following serial dilution, colonies obtained were single crossovers. The third electroporation proved more successful four blue colonies obtained following electroporation. Two gentamycin resistant, kanamycin sensitive colonies were obtained from the selection stages post electroporation. These were both double crossovers. A radioactive Southern blot was used to screen colonies (Figure 3.10).

3.3.4 Δ Rv2191

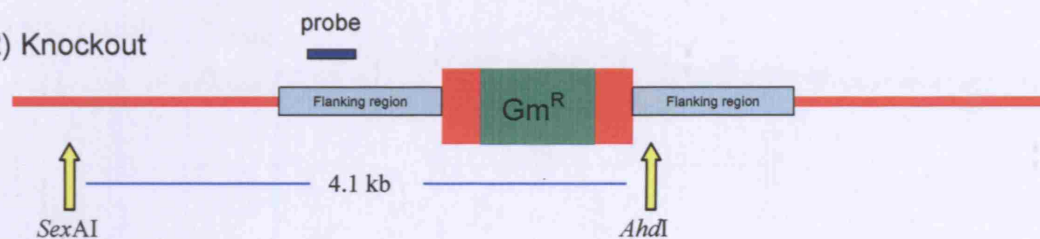
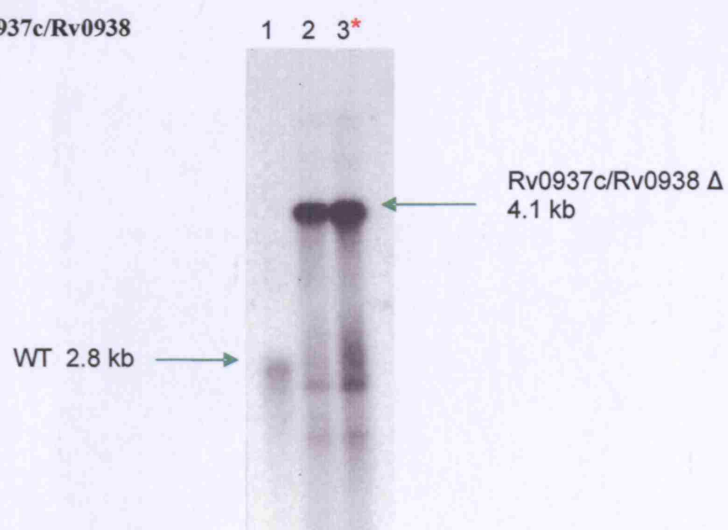
The initial electroporation was unsuccessful but the second electroporation yielded 1 blue colony. This was streaked and serially diluted and 36 white colonies were obtained at the sucrose counterselection stage. These were subsequently confirmed to be gentamycin resistant and kanamycin sensitive. Ten of these colonies were screened by non-radioactive Southern blot and all of these were double crossovers (Figure 3.11).

a) Location of the probe

1) Wild type



2) Knockout

b) Southern Blot: Δ Rv0937c/Rv0938

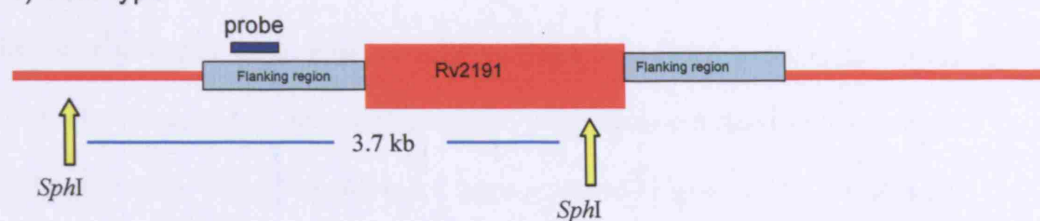
1= WT

2= Δ Rv0937c/Rv09383= Δ Rv0937c/Rv0938 (* used as Δ Rv0937c/Rv0938)**Figure 3.10: Southern blot and probe location for Rv0937c/ Rv0938 double mutant**

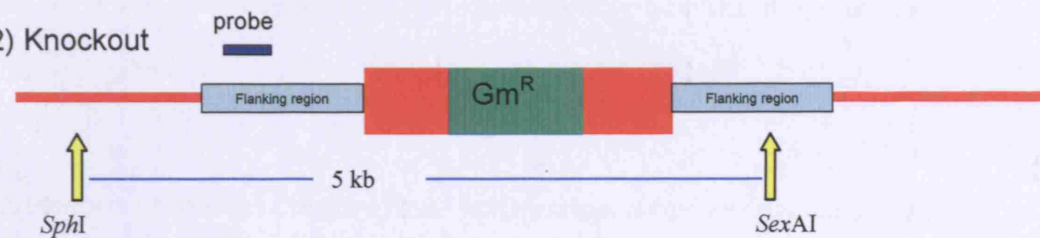
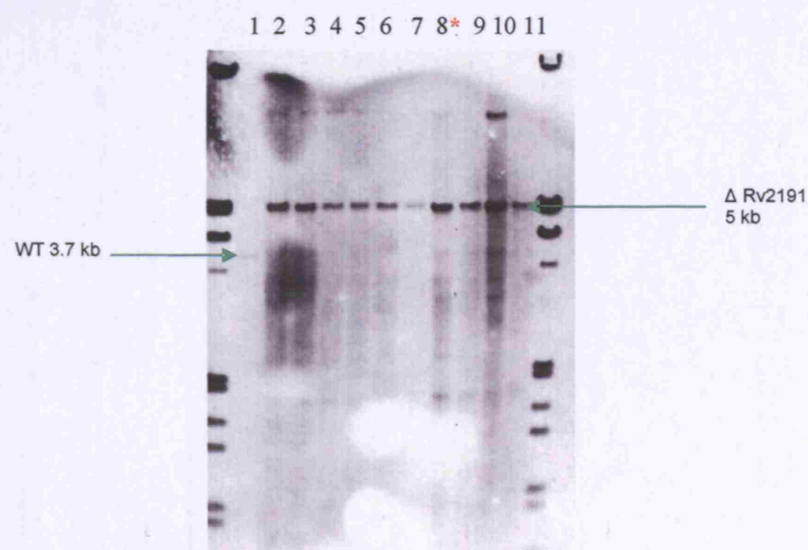
The figure shows the location of the probe with the restriction sites used to cut out the fragment used for screening double crossovers (a). Due to the insertion of the Gm^R cassette between the flanking regions of the gene, the WT and mutant will give different sized bands which can be distinguished by Southern blot (b). 3 μ g WT and 3 μ g potential double crossover DNA were digested with *SexAI* and *AhdI*.

a) Location of the probe

1) Wild type



2) Knockout

b) Southern Blot: Δ Rv2191

1= WT

2-1= Δ Rv2191 * indicates clone used as Δ Rv2191**Figure 3.11: Southern blot and probe location for Rv2191 mutant**

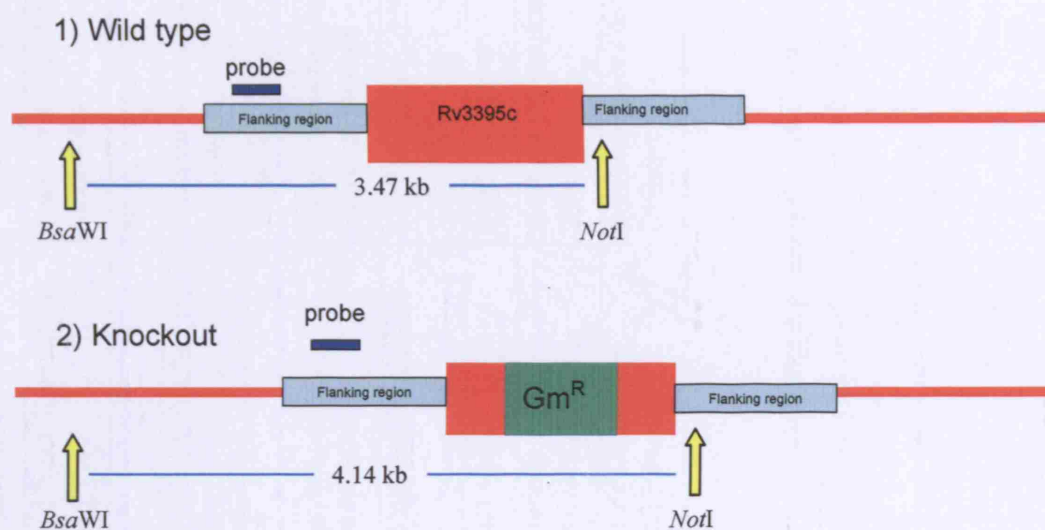
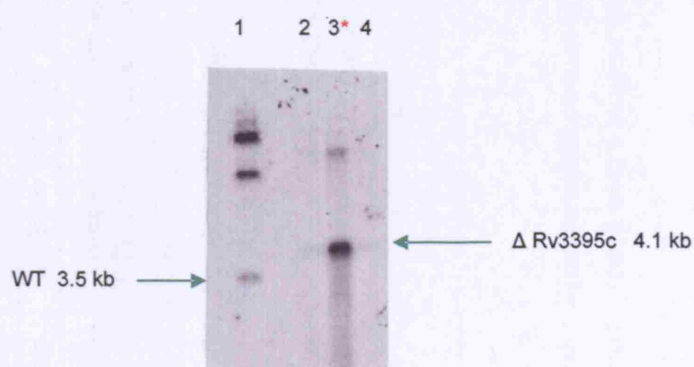
The figure shows the location of the probe with the restriction sites used to cut out the fragment used for screening double crossovers (a). Due to the insertion of the Gm^R cassette between the flanking regions of the gene, the WT and mutant will give different sized bands which can be distinguished by Southern blot (b). 3 μ g WT and 3 μ g potential double crossover DNA were digested with *SphI* and *SexAI*.

3.3.5 Δ Rv3395c

Three blue colonies were obtained following the first electroporation into Mtb. Following the sucrose counterselection stage, 16 gentamycin resistant, kanamycin sensitive colonies were obtained and 3 were screened (Figure 3.12). All of these were confirmed to be double crossovers. A radioactive Southern blot was used to screen colonies.

The numbers of colonies obtained from each electroporation, the amount of DNA used and the outcome of the screening procedures in each case are summarised in table 3.2.

a) Location of the probe

b) Southern blot Δ Rv3395c

1= WT control

2-4= Δ Rv3395c (* indicates clone used as Δ Rv3395c)**Figure 3.12: Southern blot and probe location for Rv3395c mutant**

The figure shows the location of the probe with the restriction sites used to cut out the fragment used for screening double crossovers (a). Due to the insertion of the Gm^R cassette between the flanking regions of the gene, the WT and mutant will give different sized bands which can be distinguished by Southern blot (b). 3 μ g WT and 3 μ g potential double crossover DNA was digested with *Bsa*WI and *Not*I. Δ Rv3395c

Gene	Electroporation	Colonies	Progression	Outcome
Rv0937c	1 (100 ng)	0 blue 21 white	Whites tested for spontaneous double crossovers.	Single crossovers
	2 (5 µg)	2 blue 14 white	Blues picked, streaked and serially diluted. Patched for Km sensitivity and Gm resistance.	Southern proves double crossovers
Rv0938	1 (5 µg)	0 blue 1 white	White tested for double crossover	Single crossover
	2 (2 µg)	4 blue 13 white	Blues picked, streaked and serially diluted. Patched for Km sensitivity and Gm resistance.	Southern proves double crossovers
Rv0937c/Rv0938	1 (100 ng)	0 blue 32 white	Whites tested for double crossovers	Single crossovers
	2 (1.5 µg)	1 blue 20 white	Blue colony picked, streaked and serially diluted. Patched for Km sensitivity and Gm resistance.	Colonies Km resistant. Single crossovers
	3 (5 µg)	4 blue 1 white	Blues picked, streaked and serially diluted. Patched for Km sensitivity and Gm resistance.	Southern proves double crossovers
Rv2191	1 (2 µg)	0 blue 1 white	White colony tested for double crossover	Single crossover
	2 (2 µg)	1 blue 9 white	Blue colony picked, streaked and serially diluted. Patched for Km sensitivity and Gm resistance.	Southern proves double crossovers
Rv3395c	1 (4 µg)	3 blue 2 white	Blue colonies picked, streaked and serially diluted. Patched for Km sensitivity and Gm resistance.	Southern proves double crossovers

Table 3.2: Summary table: Generation of mutant strains

The table shows the procedure involved in generating mutant strains. Time from electroporation to confirmation by Southern blot is generally 6 months. Each selection stage requires 4-5 weeks for growth of strains. Each construct was electroporated using a range of DNA quantities. For the purpose of simplicity, only the quantities yielding colonies have been shown here.

3.4 Discussion

The construction of gene knockouts in pathogenic organisms plays a vital role in furthering understanding of the pathogen and its virulence. Disrupting the gene allows us to undertake studies comparing the resultant phenotype with that of the parental wild type strain. This therefore provides a clearer idea of the importance of the encoded protein in infection models, and the information obtained in this way may have clinical significance in drug design.

To construct the gene knockouts and interrupt the function of the selected gene, a two-step system using counterselectable markers was used. In summary, an antibiotic resistance cassette (gentamycin) was inserted between the regions flanking the gene of interest in a suicide vector containing a kanamycin cassette and a counterselectable marker, the *sacB/lacZ* cassette. After transforming the plasmid DNA into wild type H37Rv, allelic replacement takes place and the mutated gene becomes incorporated into the mycobacterial DNA, replacing the original gene. This process takes place by homologous recombination.

3.4.1 Problems associated with constructing gene knockouts

Although unmarked deletions, generated through allelic exchange using non-replicating suicide plasmids are possible to achieve in Mtb, the increased screening and low efficiency make it a less practical option for generating mutants. Because of this, the mutants generated in this study were all marked with antibiotic resistance cassettes. The process of making successful gene knockouts in Mtb using counterselectable markers is, however, technically challenging,

lengthy and takes at least six months due to the slow growth of the bacteria. It is, therefore, preferable to be able to eliminate single crossovers and spontaneous mutants during the selection stages to avoid screening large numbers of false positives after such a long period. In this study, a double selection process was used with a number of different markers to aid selection.

An antibiotic resistance cassette was cloned between the two regions flanking the deleted gene. This aids in the first and final selection stage and also the final screening. Additionally, it further disrupts the gene function. As well as using an antibiotic resistance marker inside the mutated gene, a counterselectable marker was also used. In this way, double crossovers can be efficiently screened for after a series of selection stages, whereas previous methods relied solely on the delivery of the inactivated gene by the suicide vector into the Mtb genome. This approach suffered from high background due to low frequencies of double crossovers and large numbers of colonies need to be screened which is time consuming and impractical (McFadden 1996).

At the first selection stage, clones where a single crossover has taken place are identified by their antibiotic resistant growth on a selective plate. At the second selection stage, the counterselective markers are used to identify potential double crossovers which are then screened again for the loss of the antibiotic resistance carried on the vector backbone. The identity of the potential double crossovers is finally confirmed by Southern blotting.

Counterselectable markers work by promoting the death of the colonies expressing the gene. For example, the sucrose sensitivity system used here contains the *Bacillus subtilis sacB* gene which codes for levansucrase, an enzyme which converts sucrose to levans (Gay *et al.*, 1985). Expression of this gene confers sensitivity to sucrose and bacteria plated onto media containing sucrose will not survive as the levans formed in its presence is toxic for them (Steinmetz *et al.*, 1983). The *sacB* gene is in the suicide plasmid and therefore should be lost by the second selection stage. Clones which have lost the vector will not be sensitive to sucrose as Mtb does not have a *sacB* gene and is therefore sucrose resistant and able to grow on sucrose containing plates (Pelicic *et al.*, 1996).

The *sacB* gene is available in a cassette coupled with the *lacZ* gene (Parish and Stoker 2000). Expression of the *lacZ* gene confers a phenotype of blue colonies in the presence of X-gal and these colonies are easily identified in the counterselection stage where bacteria are plated onto media supplemented with 2% sucrose and X-gal. Sucrose resistant white colonies have lost the *sacB/lacZ* cassette from the original plasmid and are therefore likely to be double crossovers. The use of the *lacZ* gene allows elimination of spontaneous sucrose resistant mutants which will be blue.

3.4.2 Alternative methods for constructing knockouts

Mutants in Mtb can be made in a number of ways and another commonly used method in streptomycin resistant strains involves the *rpsL* gene (Sander *et al.*, 1995). The *rpsL* gene encodes the S12 ribosomal subunit protein target of streptomycin (Dean and Kaelbling 1981) and therefore confers sensitivity to

streptomycin. A mutation in this gene leads to streptomycin resistance and the gene works as a counterselectable marker because resistance to streptomycin is recessive (Lederberg 1951). Therefore, if copies of both wild type and mutant forms of the gene are present, the bacteria will be sensitive to streptomycin. If the bacteria harbouring single crossovers of a targeting plasmid are plated on streptomycin supplemented media, only the double crossovers will grow. A disadvantage of this method of generating mutants is that there are problems associated with working with streptomycin resistant strains, as streptomycin is one of the drugs used to treat TB.

Global gene inactivation can be achieved by inserting a fragment of DNA into random sites in the bacterial genome. Transposon mutagenesis is a useful tool for generating and screening large libraries of mutants and an advantage of this system is that it is relatively fast and huge numbers of phenotypes attributable to mutated genes can be investigated in a short space of time. However, mutating defined genes using this process is more difficult, and transposon generated mutants are not always completely stable. Making transposon insertion mutant libraries relies on phage DNA transposition complexes such as Mu (Pajunen *et al.*, 2005). These transpososomes can be used to deliver selectable markers inside the bacterial genome, thus disrupting the gene.

Transposon Site Hybridisation (TraSH), allows the identification of specific genes essential for growth (Sasseti *et al.*, 2001; Sasseti *et al.*, 2003). It has the advantage of being fast and mutations can be marked by insertions (Hamer *et al.*,

2001) but the TraSH procedure does not allow the isolation of mutants in individual genes for detailed study.

3.4.3 Concluding remarks

Making use of the various selection and counterselection stages ensured that the vast majority of the potential mutants that were screened were double crossovers. It is possible to find double crossovers at the initial selection stage after electroporation for marked mutants but this was not the case for any of the mutants obtained in this study, all of which underwent the full selection process. For fast screening of phenotypes, a technique using transposon mutagenesis may be more appropriate; however making mutations in specific genes can be difficult and the technique is not always accurate. For the rigorous phenotypic investigations undertaken in this study, making mutants using the stringent selection stages with counterselectable markers was the most suitable option.

4 Phenotype I: Primary Screening of Mutants

Hypotheses:

- 1) The mutants under study are affected in their ability to grow in a simple infection model.
- 2) The mutants under study are altered in their susceptibility to DNA damage.

4.1 Introduction

In this chapter, preliminary screening of the mutant strains constructed in the previous chapter will be undertaken to identify potential defects in both DNA repair, and in the ability to grow in a simple infection model. The first part of the chapter addresses the possibility that inactivation of the gene will affect the ability of the bacteria to grow and survive following infection. This initial screen uses the simplest infection model available, which is infection of unactivated macrophages. The second part of the chapter investigates whether the selected genes play a role in DNA repair. This will be determined using a disc diffusion susceptibility assay to examine the effects of a range of DNA damaging agents on the ability of the mutants to grow in their presence.

In the previous chapter, mutant strains of *M. tuberculosis* were isolated. These knockout strains were deficient in Rv0937c, Rv0938, Rv2191 or Rv3395c. A double mutant lacking both Rv0937c and Rv0938 was also constructed.

Once the mutants were constructed, it was decided to carry out a basic screen to investigate phenotypic variation between the strains. The first step in characterising the mutants was to look at the effect of the lack of the particular gene on growth, both *in vitro* and in a basic infection model. Although the very fact that mutants had been isolated indicated that the genes targeted were not essential, elimination of a gene may lead to a reduced growth rate *in vitro* and if so, this information would be useful in planning subsequent investigations. Furthermore, the genes inactivated may be important for survival during infection. This can be investigated by growth in macrophages and is observed as reduction in the number of colony forming units (cfu) from lysed macrophages.

Mtb is an intracellular pathogen and during inhalation of infectious particles, the bacteria are engulfed by alveolar macrophages. Following infection with Mtb, whether the individual develops clinical disease depends on their ability to mount an effective immune response against the bacteria in the macrophage and whether the bacteria can survive this. Testing survival of mutant strains in unactivated macrophages emulates the early stages of infection, prior to activation by T cell mediated immunity. In comparison, investigating survival in activated macrophages represents survival at later stages of infection, following macrophage activation by T helper cells. For thorough investigation, it is important to look at survival in activated and unactivated macrophages. However, for the purpose of a basic screen, it was decided to investigate survival of mutants in unactivated macrophages. It must be added that simply harvesting and treating the macrophages for infection does result in activation to some degree.

The phenotype of the mutant strains was also investigated by examining bacterial growth in the presence of DNA damaging chemicals by means of a disc diffusion assay. This is a relatively crude and artificial approximation of DNA damaging conditions as may be induced by the macrophage during infection *in vivo*. The phenotype of strains can be determined by measuring their growth in the presence of various antibiotics or DNA damaging agents. This assay determines susceptibility of a strain to the compound of interest as the zone of inhibition of growth around an impregnated disc. Differences observed in this measurement of the zone of inhibition reflect differences in the concentration of the agent required to prevent growth. This is due to the fact that a concentration gradient of the chemical is formed by diffusion from the impregnated disc.

4.2 Growth in vitro

Growth *in vitro* was investigated to look for any differences in growth in culture between the knockouts and wild type and to screen for any marked phenotype. The main limitation of examining growth *in vitro* is that it is not representative of conditions *in vivo*. However, it is nonetheless a useful screen for any growth phenotypes and can be used to pinpoint deficiencies or increases in growth. Investigating growth *in vitro* is a useful marker against which to measure growth *in vivo*. It allows us to determine whether a potential decrease in growth *in vivo* simply reflects a growth impairment of the strain, seen as a reduced ability to grow, or is attenuation specific to infection.

Growth *in vitro* was measured in rolling cultures using Dubos media with a starting OD₆₀₀ of close to 0.01. Growth was measured as the increase in optical density over a period of 12 days. Experiments were performed in duplicate (Figures 4.1 and 4.2).

None of the NHEJ or Rv2191 mutant strains appeared to have a growth phenotype *in vitro*. This is to be expected as the genes under study are not predicted to be involved in growth under standard conditions without the presence of excessive DNA damage. During conditions of growth in culture, there is limited opportunity for DNA damage to arise, other than that caused as a result of normal DNA replication and therefore limited opportunity for repair. A slight difference in growth was observed between the Rv3395c mutant and wild type strains between days 5 and 8. However this was corrected by day 9, where the mutant growth reached the same level as that of the wild type.

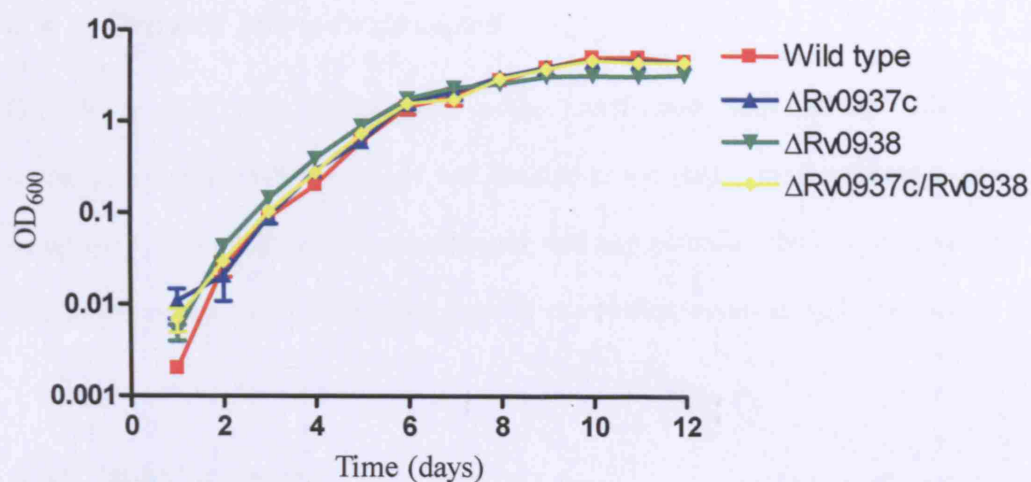


Figure 4.1: Growth of the NHEJ mutant strains *in vitro*

The graph shows the *in vitro* growth of the NHEJ mutant strains ($\Delta Rv0937c$, $\Delta Rv0938$ and $\Delta Rv0937c/Rv0938$) compared to a wild type control over 12 days. The differences in growth between the wild type and mutant strains are not statistically significant.

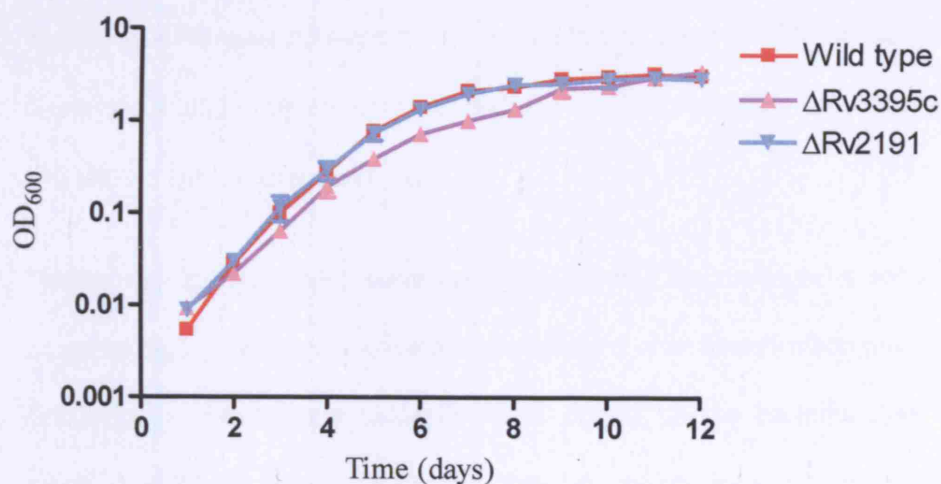


Figure 4.2: Growth of the Rv2191 and Rv3395c mutant strains *in vitro*

The graph shows the *in vitro* growth of the $\Delta Rv2191$ and $\Delta Rv3395c$ strains compared to a wild type control over 12 days. The difference in growth between the wild type and Rv2191 mutant strain is not statistically significant as determined by an Unpaired T test. There is a statistically significant difference in growth between the Rv3395c mutant strain and the wild type at time points between 5 and 8 days (Unpaired T test, d5 $p=0.004$, d6 $p=0.0019$, d7 $p=0.0015$, d8 $p=0.0451$).

4.3 Growth in macrophages

Growth *in vivo* was investigated using unactivated bone marrow derived macrophages from Balb/c mice. It was decided to use Balb/c mice as these are the breed used for *in vivo* animal experiments, and any potential phenotypes could be further investigated by investigating growth in a murine model using Balb/c mice.

4.3.1 NHEJ mutants

The growth of the NHEJ mutants in unactivated macrophages followed a similar pattern of growth as the wild type with no mutants being significantly affected by the end of the time course (Figure 4.3). However, at day 3 there is a statistically significant difference between wild type and mutant strains, with the mutant strains displaying a slight impairment in growth ($\Delta Rv0937c$ $p=0.0006$; $\Delta Rv0937c/Rv0938$ $p=0.0009$). This is corrected by day 10.

Despite the fact the environment inside unactivated macrophages is not thought to be as damaging as that of activated macrophages, unactivated macrophages would still be expected to have some cytotoxic effects on the bacteria. Any damage incurred, although potentially limited, may be repaired by alternative DNA repair pathways or proteins.

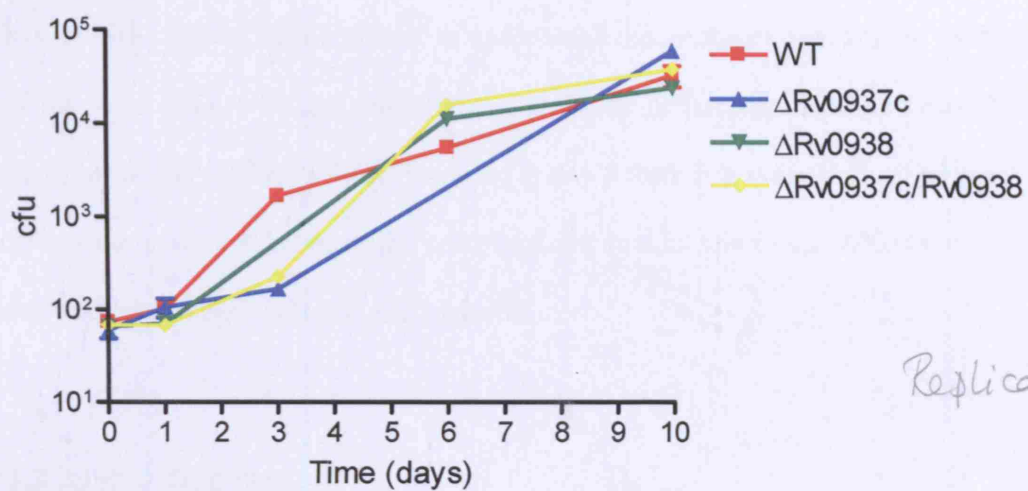


Figure 4.3: Growth of NHEJ mutant strains in unactivated macrophages

There is no statistically significant difference between mutant and wild type strains at day 10, as determined by an Unpaired T test. At day 3, there is a statistically significant difference in growth between wild type and $\Delta Rv0937c$ ($p=0.0006$) and $\Delta Rv0937c/Rv0938$ ($p=0.0009$) strains.

4.3.2 Rv2191 mutant

The growth of the Rv2191 mutant in unactivated macrophages was similar to that of the wild type, with no statistically significant differences seen between the strains after day 3 (Figure 4.4). However, at day 1 there is a statistically significant difference in growth between the wild type and mutant strains ($p=0.0034$), which may indicate a slight defect in early growth.

4.3.3 Rv3395c mutant

In unactivated macrophages, the Rv3395c mutant and the wild type grew to the same extent by the end of the time course at day 10 (Figure 4.5). However, a statistically significant difference in growth was seen between the wild type and the mutant at day 3 ($p= 0.0002$) and day 6 ($p=0.0009$), as determined by an unpaired T-test, with the mutant strain exhibiting higher cfu than the wild type. Thus, it would appear that the Rv3395c mutant is actually able to grow faster in unactivated macrophages than the wild-type strain, until it enters a plateau phase. The wild type strain does reach the same level of growth, but at a later time.

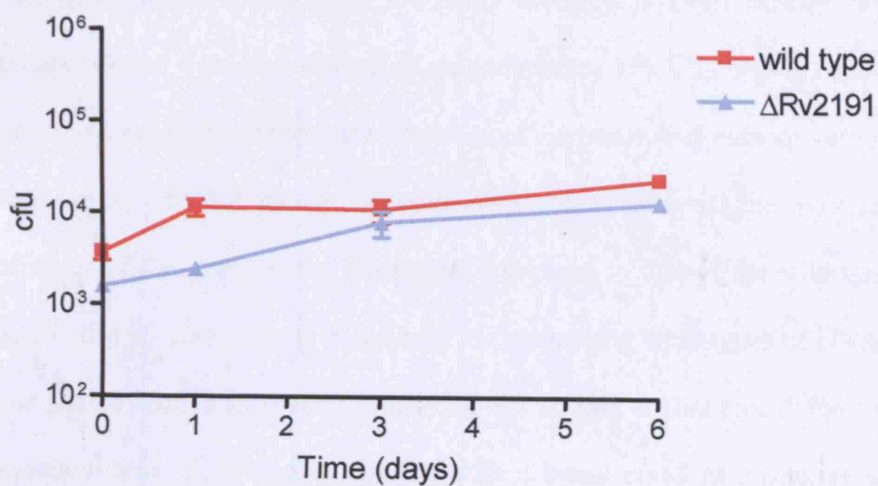


Figure 4.4: Growth of the Rv2191 mutant strain in unactivated macrophages

There is no difference between the mutant and wild type strain as determined by an Unpaired T test after day 3. At d1, there is a statistically significant difference in growth between the mutant and wild type strains ($p=0.0034$).

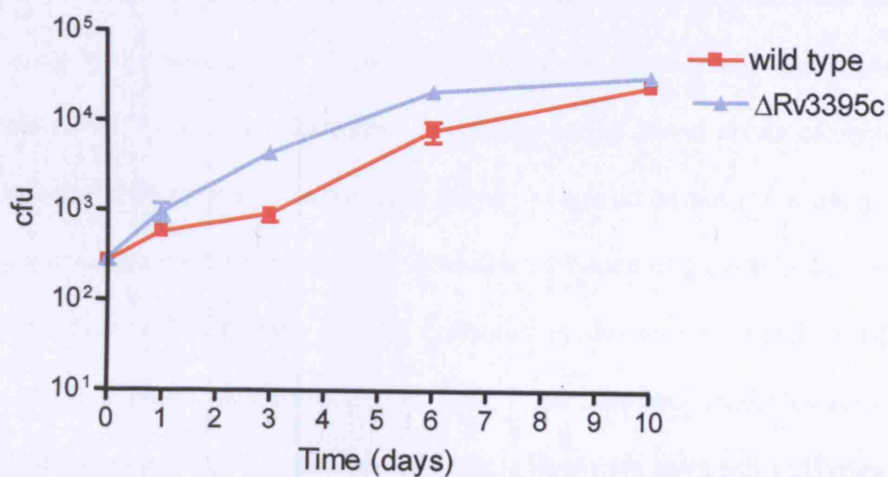


Figure 4.5: Growth of the Rv3395c mutant strain in unactivated macrophages

There is no difference between the mutant and wild type strain as determined by an Unpaired T test at d10. There is a statistically significant difference between strains at d3 ($p=0.0002$) and d6 ($p=0.0009$).

4.4 Disc Diffusion Susceptibility Assay

The genes under investigation are either involved in DNA damage repair as a component of a repair pathway or are induced by DNA damaging conditions. In order to gain an insight into the functions of the genes, the mutants were subjected to a variety of DNA damaging conditions *in vitro* to investigate the effect of the absence of the gene on the phenotype compared to that of the wild type. These susceptibility assays are a useful tool in determining what types of DNA damage the gene might be involved in repairing and so give a vital clue to the role of the mutated gene. Phenotyping mutants with a broad range of damaging agents *in vitro* alongside looking at growth in an infection model can provide us with a clearer idea of the role of the gene in repairing specific kinds of damage.

In order to screen the mutants for their susceptibility to DNA damaging conditions, thus investigating their role in DNA repair, a disc diffusion sensitivity assay was used (Bauer *et al.*, 1966). This is a relatively crude screen for susceptibility to DNA damaging conditions and is based on an estimate of the effect of the compound on bacterial growth on a solid medium. The assay gives an approximate measurement for any potential inhibition of growth in the presence of the compound of interest. This is measured by the zone of inhibition of growth around a paper disc impregnated with a standardised concentration of DNA damaging agent. The disc diffusion assay is used frequently as a screening tool for measurement of susceptibility of mutants (Darwin and Nathan 2005; Dayaram *et al.*, 2006). Clinically, the assay is used to measure the susceptibility or resistance of a strain to a specific antibiotic. In this way, the treatment can be tailored to the individual patient as the relationship between the Minimum Inhibitory

Concentration (MIC) and the zone of inhibition can be calculated, allowing a precise dose of antibiotic to be administered. As the distance from the disc increases, there is a logarithmic decrease in antibiotic concentration. To utilize this, standardised regression curves are available to link the size of the zone of inhibition to the MIC of the antibiotic. The exact degree of susceptibility of a strain can be measured using E tests, a commercial tool which is a way of quantifying the zone and linking this to the MIC of the drug in question.

An alternative method for measuring susceptibility to DNA damaging agents involves growing up a culture to mid-exponential phase and then inoculating it with a standardised concentration of the damaging agent, incubating it for a set time period, for example 24 hours, and then serially diluting and plating. After 2 week's growth, colony numbers can be compared between wild type and mutants. Although this method is accurate for this purpose and is a very good representative means of quantitatively assessing the viability of mutants after exposure to damaging agents, it is very time consuming and impractical for a screen, due to the number of cultures required per strain per stress. Therefore for screening large numbers of mutants with a variety of DNA damaging agents, discs are a more viable option and allow a large number of repeats and so provide more reproducible data. However, it must be noted that although these disc diffusion assays are a good screen, they measure susceptibility to DNA damage as lack of growth, rather than viability following exposure to DNA damaging conditions. They do not distinguish between a bacteriostatic and a bactericidal effect. Any potential phenotype would have to be followed up and confirmed using a more rigorous test. Despite this, they are often used as a conclusive tool for measuring

the effect of specific damaging agents on mutant strains of Mtb (Manganelli *et al.*, 2001; Darwin and Nathan 2005).

4.4.1 Standardisation

Initially tests were carried out to standardise the assay. The standardisation was designed to determine the optimum quantity of compound, which, when applied on 6mm filter discs (Oxoid), would result in a suitably sized zone of exclusion in the wild type strain. A range of quantities of each compound were tested (Table 4.1) and the most suitable were chosen for further use. It must be noted that not all compounds are suitable for use in a disc diffusion assay, often due to poor solubility. The compounds tested were mitomycin C, ofloxacin, bleomycin and menadione. The compounds were chosen because of their range of actions.

Table 4.1: Standardisation of disc diffusion susceptibility assay

Quantities in bold indicate the quantities that were used for further investigations.

Compound	Quantity 1	Quantity 2	Quantity 3
mitomycin C	10 µg	20 µg	40 µg
ofloxacin	2 µg	4 µg	7.5 µg
bleomycin	5 µg	7.5 µg	10 µg
menadione	40 µg	70 µg	100 µg

For each of the compounds used, increasing the dose resulted in a larger zone of inhibition of growth, as might be expected (Figure 4.6). The quantity that gave approximately an 8-15mm zone of inhibition around the disc was chosen for subsequent investigations. This was decided to be the optimum concentration as it was hoped that differences between the wild type and the mutants would be easy to measure. Obviously, the larger the zone, the more accurate the measurement but due to the constraints on plate numbers and in order to generate the maximal number of repeats for statistical analyses, concentrations with zones of inhibition around 10mm were chosen because they would allow 3 discs per plate, with enough space between them to be able to fully assess the zone of inhibition, even allowing for potentially larger zones with mutants that were more susceptible to the agent being tested.

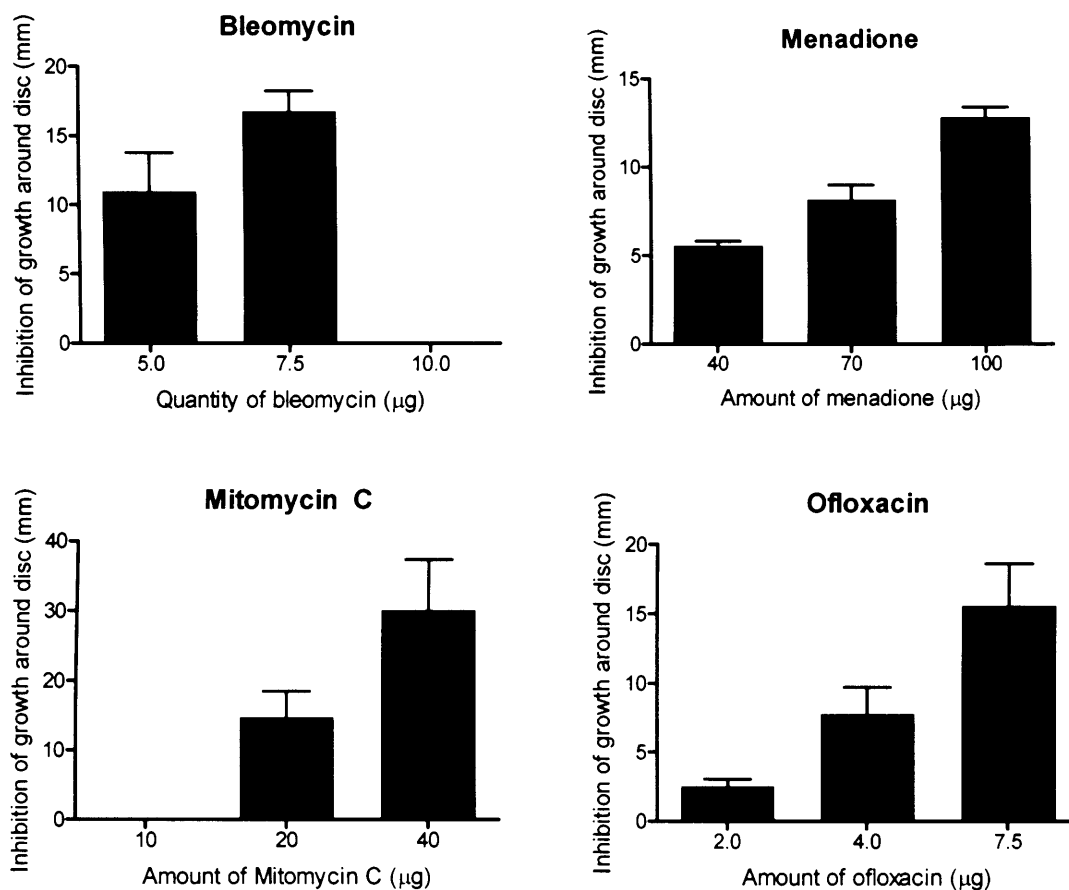


Figure 4.6: Standardisation of the disc diffusion susceptibility assay.

The mean diameter of the zone of growth inhibition for the wild type strain is shown for 3 quantities of each agent. Each measurement is the mean of 2 separate experiments, each with 3 technical replicates (the 3 discs on each plate). Standard error is indicated by the error bars.

At 10 μg bleomycin (above left), bacterial growth was completely inhibited

At 10 μg mitomycin C (below left), there was no inhibition of growth observed.

4.4.2 Disc Diffusion Assays

Once the standardisation was complete, disc diffusion assays were used to assess the relative susceptibility of the mutant strains compared with the wild type to the selected DNA damaging agents (Figure 4.7). Each experiment was performed with 3 biological replicates, each with 3 discs per plate, and 2 technical replicates. In this way, maximal data was generated for performing statistical analyses. *Mtb* was grown up to exponential phase with an OD₆₀₀ of 0.3-0.4 and 50µl spread onto a 7H11 agar plate using a cotton swab to ensure even distribution. Once the bacteria were absorbed into the agar plate (30 min), 3 impregnated discs per plate were placed on the surface, equidistant from each other. The discs were prepared approximately 12 h before the experiment and allowed to dry overnight (in the dark) in a Petri dish. The plates were incubated at 37°C for 12 days. The diameters of the zones of inhibition surrounding the disc were measured and then compared against the wild type data to identify statistically significant differences between strains.

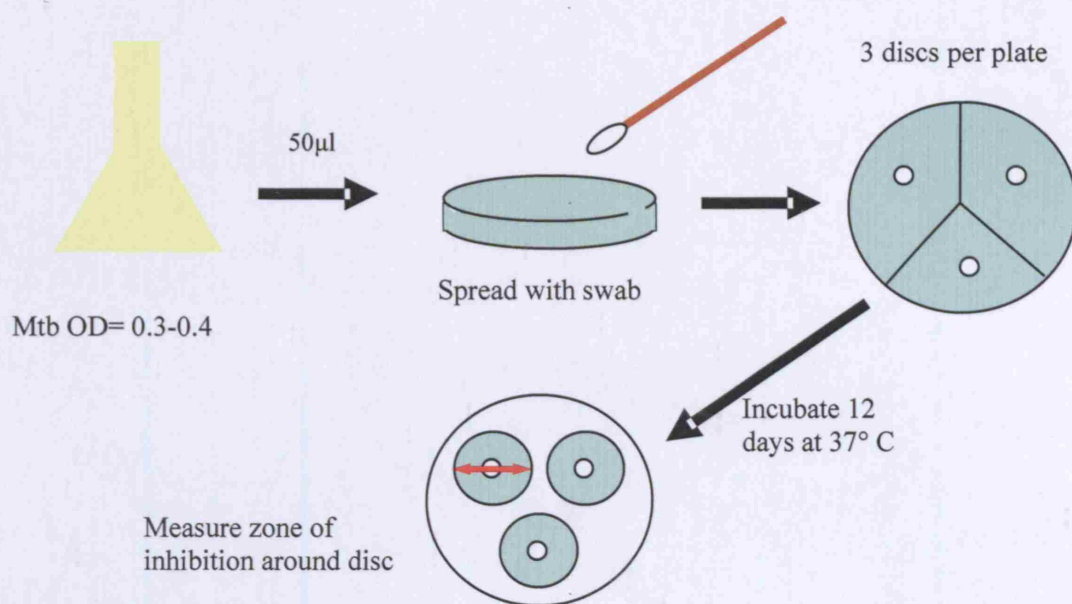


Figure 4.7: Disc diffusion assay methodology

Schematic of the disc diffusion assay performed on mutant and wild type strains of Mtb to determine susceptibility to a range of DNA damaging agents. Each Mtb culture was grown to an OD₆₀₀ of 0.3-0.4 and 50µl spread on each plate using a swab to ensure even distribution. Three 6mm paper discs (Oxoid), impregnated with the selected DNA damaging agent, were placed on each plate, equidistant from each other. Plates were incubated for 12 days at 37°C and the diameters of the zones of inhibition of growth were measured.

4.4.2.1 Mitomycin C

Mitomycin C is an antibiotic derived from *Streptomyces caespitosus*. It acts as an alkylating agent of DNA and has been reported to be antineoplastic and mutagenic (Szybalski 1958). Mitomycin C is a bifunctional alkylating agent, with 2 reactive groups, and is thus able to react with 2 sites in the DNA. It is this action which can lead to complementary strands becoming crosslinked (Iyer and Szybalski 1963). The reactive sites occur predominantly in guanine residues (Vidal *et al.*, 2006) and exposure can lead to both mitomycin C-guanine monoadducts and biadducts. These can occur as either interstrand or intrastrand crosslinks (Palom *et al.*, 2002), resulting in double strand breaks. It has been shown that the reactivity of specific bases depends on a number of factors including the flanking bases (Richardson and Richardson 1990) and steric position (Rich *et al.*, 1984). Inter-strand crosslinks result if the 2 sites are on opposite strands, and can completely prevent DNA synthesis and inhibit transcription. For this reason, mitomycin C is used in chemotherapy to treat cancer (Iyer and Szybalski 1963), where it acts specifically on tumour cells, owing to their fast replication rate compared with normal cells, resulting in inhibition of their growth.

In bacteria, exposure to mitomycin C ultimately leads to impaired survival and decreased viability. In *E. coli*, repair of mitomycin C induced DNA crosslinks occurs primarily via nucleotide excision repair and homologous recombination working together (Dronkert and Kanaar 2001). RecA additionally plays an essential role, while a significant contribution to the repair of mono-adducts is provided by nucleotide excision repair alone (Ramos *et al.*, 1998).

The results of the disc diffusion susceptibility assays using mitomycin C revealed that the Rv3395c mutant showed the highest susceptibility to this compound, with the zone of inhibition of growth being almost twice that of the wild type (Figure 4.8). This difference was statistically significant (unpaired T test $p < 0.0001$). The size of the zone of inhibition for the Rv0937c/Rv0938 double mutant also was statistically significantly different from that of the wild type ($p = 0.0286$) revealing increased susceptibility to mitomycin C, but the Rv0937c and Rv0938 single mutants and the Rv2191 mutant did not show a statistically significant difference in susceptibility compared with the wild type. This observation suggests that the complete inactivation of the NHEJ pathway in the double mutant has a more profound effect on the ability of the bacteria to survive at least some kinds of DNA damage than loss of individual components of the pathway.

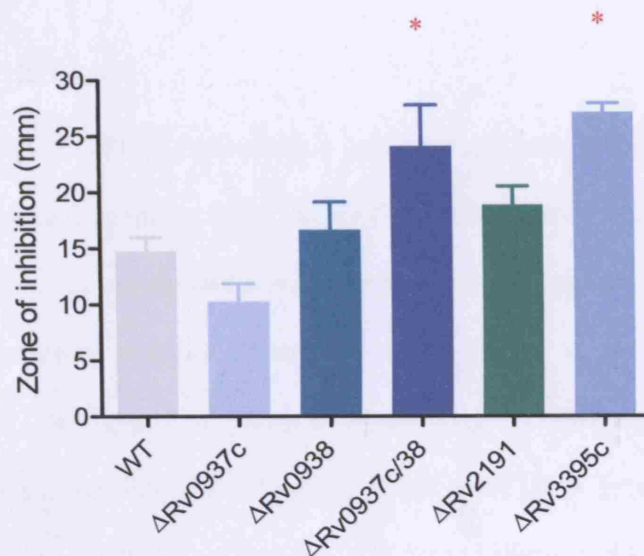


Figure 4.8: Disc diffusion assay showing susceptibility of mutant strains to mitomycin C.

Mitomycin C is a DNA damaging agent that induces alkylation and inter-strand crosslinks in bacterial DNA. Susceptibility was measured as diameter of the zone of inhibition of growth around a 6mm paper disc impregnated with 20 μ g mitomycin C. An asterisk denotes a statistically significant difference from the wild type as determined by an Unpaired T test.

(Rv0937c/Rv0938 $p=0.028$; Rv3395c $p<0.0001$)

4.4.2.2 Ofloxacin

Ofloxacin, an analogue of nalidixic acid, is a synthetic broad spectrum quinolone antibiotic used against a wide range of susceptible microorganisms such as *Mtb*, *Staphylococcus aureus* and *Bordetella pertussis* among others. Ofloxacin inhibits DNA synthesis and the bactericidal effect is due to its potent inhibition of bacterial DNA gyrase, an enzyme which controls DNA supercoiling. Ofloxacin prevents supercoiling and relaxation reactions of DNA gyrase by binding to the bacterial DNA (Shen and Pernet 1985) and affecting DNA strand breakage and reunion. A complex is formed with the drug and gyrase subunitA covalently binding to the DNA ends (Higgins *et al.*, 1978) and inhibiting religation of the broken strand, resulting in the formation of bulky adducts and a double strand break. DNA replication is therefore inhibited. When *Mtb* are exposed to a low dose of ofloxacin, survival is impaired as DNA is damaged and must be repaired.

In the disc diffusion assay, the Rv2191 mutant showed a statistically significant increase in susceptibility to ofloxacin compared to the wild type ($p=0.0091$) (Figure 4.9). This observation supports a potential role for Rv2191 in DNA damage repair in *Mtb*. Although the Rv0937c/ Rv0938 double mutant and the single Rv0937c and Rv3395c mutants did show a slight increase in sensitivity on exposure to ofloxacin compared with the wild type, these differences were not statistically significant.

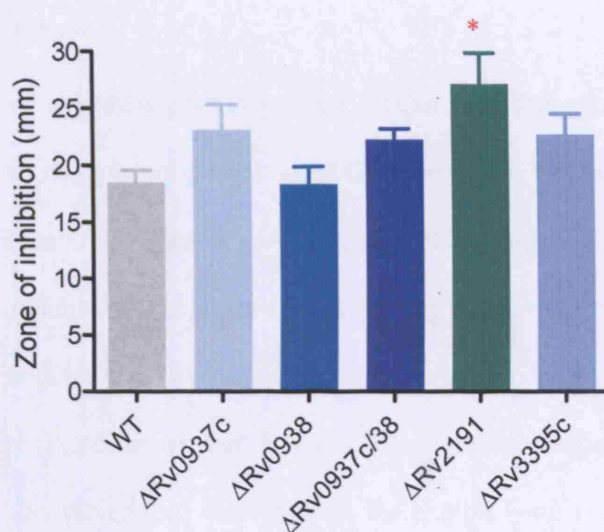


Figure 4.9: Disc diffusion assay showing susceptibility of mutant strains to ofloxacin.

Ofloxacin is an antibiotic that inhibits bacterial DNA gyrase. Susceptibility was measured as diameter of the zone of inhibition of growth around a 6mm paper disc impregnated with 4 μ g ofloxacin. Rv2191 displayed a statistically significant difference in growth compared to the wild type as determined by an Unpaired T test, $p=0.0091$.

4.4.2.3 Bleomycin

Bleomycin is an antineoplastic antibiotic from *Streptomyces verticillus* which is active against most Gram-positive and Gram-negative bacteria by causing double strand breaks in DNA (Dedon and Goldberg 1992). Although its exact mechanism of action is unknown, it is believed that bleomycin prevents thymidine uptake into DNA and labilizes the DNA structure (Suzuki *et al.*, 1969), causing abasic sites with closely opposed strand breaks. The double strand breaks caused by bleomycin are thought to be repaired by homologous recombination or non-homologous end joining repair mechanisms. Bleomycin is commonly used as a chemotherapeutic agent where it acts preferentially on rapidly dividing tumour cells and induces the p53 tumour suppressor gene. The p53 tumour suppressor gene is involved in cell cycle arrest, preventing replication of damaged DNA and ultimately tumour formation (Friedberg *et al.*, 1995).

The results of the disc diffusion assay revealed that all NHEJ mutants were significantly more susceptible to bleomycin than the wild type (Figure 4.10), as determined by an unpaired T-test ($\Delta Rv0937c$ $p=0.00020$, $\Delta Rv0938$ $p=0.0026$, $\Delta Rv0937c/38$ $p<0.0001$). The difference in susceptibility between these strains was similar. There was no significant difference between either the Rv3395c mutant, or the Rv2191 mutant and the wild type. As mentioned previously, double strand breaks are known to be repaired by HR and NHEJ in eukaryotes so the observation that the NHEJ mutants showed an increase in susceptibility to this kind of damage supports a similar role for NHEJ in Mtb.

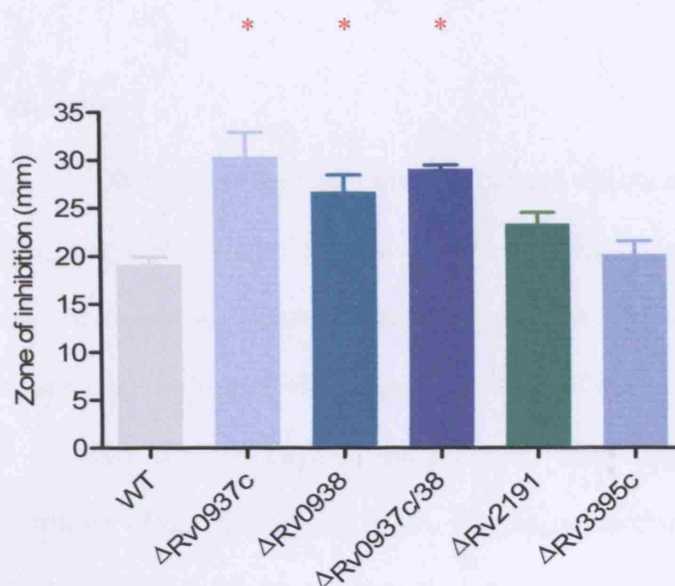


Figure 4.10: Disc diffusion assay showing susceptibility of mutant strains to bleomycin.

Bleomycin is an anti-neoplastic antibiotic that induces double strand breaks in bacterial DNA. Susceptibility was measured as diameter of the zone of inhibition of growth around a 6mm paper disc impregnated with 7.5 μ g bleomycin. An asterisk denotes a statistically significant difference from the wild type as determined by an Unpaired T test. All NHEJ mutants displayed increased susceptibility compared to the wild type strain. (Δ Rv0937c $p=0.0020$, Δ Rv0938 $p=0.0026$, Δ Rv0937c/Rv0938 $p<0.0001$).

4.4.2.4 Menadione

Menadione is a DNA damaging agent that mimics the effects of oxidative stress by initiating enzymatic redox cycling. *In vivo*, menadione is enzymatically reduced by one-electron transfer reactions prior to auto-oxidation, which generates superoxide radicals (Friedberg *et al.*, 1995). Menadione also causes the formation of other reactive oxygen species such as hydrogen peroxide and hydroxyl radicals which damage the DNA. Arylation reactions are caused via modification of biomolecules by the menadione ring structure itself (Eaton 2006). The type of radical generated is an important determinant of the resulting damage and in order to survive this exposure to reactive oxygen species, the bacteria must be able to repair the damaged DNA.

The disc diffusion assays indicated that the Rv2191 mutant and the Rv0937c/Rv0938 double mutant were more susceptible to menadione than the wild type (Figure 4.11), with the difference being statistically significant (Δ Rv2191 $p=0.0002$, Δ Rv0937c/Rv0938 $p=0.0172$). Menadione induces a range of damage to the bacterial DNA, which could potentially be repaired by a number of pathways. As well as repair pathways, ROI can be eliminated by a number of antioxidant enzymes such as catalase and the number and breadth of DNA repair systems highlights the importance of maintaining the integrity of the DNA. A number of repair pathways including NER and NHEJ are thought to play a role in the repair of the damage induced by oxidative stress, which might explain why these mutants had increased susceptibility. The fact that the double Rv0937c/Rv0938 mutant was more susceptible to than the single mutants, may imply that if one component of the pathway is missing, it is impaired but still

functioning to a lesser extent. Once the entire pathway is knocked out, its function is presumably entirely inhibited.

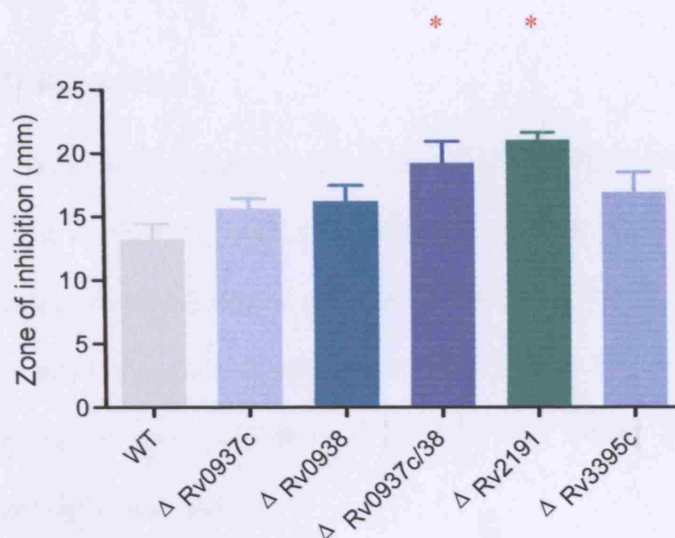


Figure 4.11: Disc diffusion assay showing susceptibility of mutant strains to menadione.

Menadione is a redox recycling agent that causes oxidative damage. Susceptibility was measured as diameter of the zone of inhibition of growth around a 6mm paper disc impregnated with 70μg menadione. An asterisk denotes a statistically significant difference from the wild type as determined by an Unpaired T test. The Rv0937c/Rv0938 and Rv2191 mutants were significantly different from the wild type strain (Δ Rv0937c/Rv0938 $p=0.0172$, Δ Rv2191 $p=0.0002$).

4.5 Discussion

None of the mutants displayed a major growth deficiency *in vitro* which was to be expected. It has recently been demonstrated that Msm Δ Rv0938 is not essential for growth in culture (Gong *et al.*, 2004). It was also shown that in *B. subtilis*, a *ku*-like mutant, a *lig*-like mutant and a *ku-lig* like double mutant displayed wild type growth *in vitro* (Weller *et al.*, 2002). The results from this study are consistent with these findings.

Although there were some differences in growth between selected mutants and the wild type strain in the early stages of infection, none of the mutants showed impaired growth in unactivated macrophages by the end of the time course. These findings suggest that these specific genes play a minimal role in growth under non-DNA damaging conditions, where perhaps other mechanisms repair the limited amount of damage sustained here. This could be investigated further by assessing growth in activated macrophages, where the bacteria are exposed to RNI and ROI. Although the use of macrophages allows investigation into the growth of mutants in the early stages of infection, it has been observed that growth in macrophages does not always correlate to viability *in vivo* in mice (Stewart *et al.*, 2002). Therefore the phenotypes of the mutant strains should be investigated in mice as well.

Bacterial growth was greatly affected by exposure to various DNA damaging agents on filter discs in the disc diffusion assays. The NHEJ mutants showed an interesting response and greatest affected was the Rv0937c/Rv0938 double mutant. This showed a statistically significant increased susceptibility to bleomycin, menadione and mitomycin C, suggesting a role for the pathway in the

repair of damage induced by these compounds. The single mutants were less susceptible and only in the presence of bleomycin was their growth decreased compared with the wild type. Bleomycin acts by inducing double strand breaks and labilising the DNA, so it is not surprising that the NHEJ mutants show an increased susceptibility to this agent, as NHEJ is one of the primary mechanisms for repairing double strand breaks, along with homologous recombination.

Mitomycin C damages the DNA by forming inter-strand crosslinks and it is possible that after exposure to low doses, when these are removed by excision processes, the DNA becomes prone to breakage. The results suggest that these breaks are then at least in part repaired by the NHEJ pathway. When the NHEJ pathway is removed, the ability of the mutant to repair this damage is impaired, implicating NHEJ. The fact that upon exposure to mitomycin C, the single NHEJ mutants did not show a statistically significant decrease in growth, suggests that a different repair mechanism may be taking over or that the components of the pathway link up to another pathway. Alternatively, there could be different proteins that can interact with the remaining component of the pathway to allow it to work. For example, it has been proposed that an alternative ATP-dependent ligase can fulfill some of the roles of Rv0938 during repair by NHEJ in *Msm* (Gong *et al.*, 2005).

The Rv0937c/Rv0938 double mutant was significantly more susceptible to menadione compared with the wild type. Menadione causes superoxide stress and induces damage by exposing the bacteria to reactive oxygen intermediates, leading to a range of damage including double strand breaks. The fact that the

single mutants were not significantly affected by exposure to menadione may again suggest that in the single mutants, the pathway can either function with one missing component or, as suggested previously, that it may use additional components to repair the damage. In the double mutant, the entire NHEJ pathway was removed, thus relying on an alternative repair mechanism such as homologous recombination to repair the damage. The fact that the zone of inhibition is increased in the double mutant may suggest that HR and NHEJ work together to repair damaged strands. This could be further investigated with a RecA/Rv0937c/Rv0938 mutant.

Overall, the results from the disc diffusion assay suggest a role for NHEJ in the repair of double strand breaks. This is supported by recent work (Gong *et al.*, 2005), and could be further explored by experiments looking to characterise and specify the type of repair and the deficiencies of these mutants. It would also be of interest to investigate the growth of the mutants in more sophisticated models of infection.

The Rv2191 mutant showed significantly increased susceptibility to menadione and ofloxacin. As described previously, the damage incurred by exposure to menadione is similar to damage incurred by exposure to reactive oxygen intermediates, which cause a range of damage to the DNA, including single and double strand breaks. Therefore, it is possible that Rv2191 is involved in repairing damage caused by exposure to ROI.

It has been demonstrated that UvrB has a role in defence against the products of phox *in vivo* (Darwin and Nathan 2005) and therefore a potential role in the survival against oxidative stress. However, the same study reported a discrepancy between these observations and the results of a disc diffusion assay. This may be explained if the oxidative damage inducing agents used *in vitro* are not representative of damaging conditions *in vivo*. There are many other factors associated with the host immune response *in vivo*. However, this reported discrepancy does give weight to the idea that the disc diffusion assay is only a basic screen and *in vitro* experiments are not always indicative of results obtained *in vivo*. It must also be noted that the response of a strain to specific generators of ROI can vary as compounds generate ROI/ RNI using slightly different mechanisms, leading to differences in their effects. The fact that the Rv2191 mutant displayed increased sensitivity to a chemical inducing ROIs, coupled with the recent published findings detailed above, support a role for the gene and potentially for the NER pathway as a whole, in the repair of DNA damage sustained as a result of exposure to ROI and/or RNI.

Interestingly, the Rv2191 mutant did not display increased susceptibility to mitomycin C, despite the fact that the crosslinks which it induces, are predicted to be repaired by homologous recombination and nucleotide excision repair (Dronkert and Kanaar 2001). It is possible that the role of Rv2191 is replaced by UvrC here, and that the actual strand breaks caused are repaired by alternative pathways.

Exposure to ofloxacin, an inhibitor of DNA gyrase, also inhibited growth of the Rv2191 mutant strain compared with the wild type. The decreased ability of Rv2191 to repair damage caused by ofloxacin may be related to the fact that in *E. coli*, Cho (which shows homology to Rv2191) improves the efficiency of the excision of bulky adducts (Moolenaar *et al.*, 2002) and exposure to ofloxacin is likely to cause the formation of such bulky adducts. This is due to the fact that it covalently links DNA gyrase to the DNA, a process which also leads to double strand breaks as a consequence of trapping an intermediate of the normal reaction pathway. Cho is thought to be an additional, but not essential component of NER that works alongside UvrC. If Rv2191 plays a similar role to Cho, then the increased susceptibility of the mutant strain observed in these disc diffusion assays suggests that UvrC alone is not capable of repairing the damage sustained. However, it is possible that UvrC does repair much of the damage but for optimal repair, Rv2191 is required in addition. It is also possible that Rv2191 may work in a different way from Cho, as Rv2191 contains an additional exonuclease domain which is not present in Cho. This raises the possibility that it could incise then degrade the DNA rather than relying on UvrC to make a second incision. Rv2191 is clearly only important for the repair of certain kinds of DNA damage, which explains why the mutation is not lethal.

The Rv3395c mutant showed a significantly increased susceptibility to mitomycin C only. Rv3395c contains a RecA-like structural domain which has been linked to motor function (Ye *et al.*, 2004) and is found in a number of proteins with a known function in DNA repair. It seems likely, given that Rv3395c is upregulated

under DNA-damaging conditions, that it could use its RecA-like domain for mechanical work involved in repair. This result supports that hypothesis.

4.5.1 Future work

The work in this chapter was a basic screen to look for indications of a role in DNA repair for the mutants under study. In order to confirm any findings, more rigorous experiments would have to be performed. The NHEJ and Rv2191 mutants showed potentially interesting phenotypes in the disc diffusion assays where growth was inhibited by more than one DNA damaging agent. This suggests a role for these genes and presumably the pathways they are part of, in DNA repair, and potentially during survival *in vivo*. As the Rv3395c mutant only showed impaired growth in the presence of mitomycin C, it was decided to focus this study on the other mutants constructed, in order to more fully pursue the investigation into the phenotypes of those mutant strains.

The next stage of this work would be to investigate the phenotypes of the selected strains in further detail. The limitations of the disc diffusion assay have been mentioned before, and to confirm and further investigate the effect of mutating these genes on susceptibility to DNA damage, viability following exposure to DNA damaging agents should be investigated. This would distinguish whether the DNA damaging agent was affecting growth or viability and hence whether it had a bactericidal or bacteriostatic effect on the mutants.

To confirm these initial results, the effects of additional DNA damaging agents, similar to those which resulted in heightened mutant sensitivity, could be

investigated. Thus, the effects of alternative oxidative damage inducing agents, such as t-butyl hydroperoxide could be explored. The effects of nitrosative stress could also be investigated by determining viability following exposure to compounds such as acidified sodium nitrite or S-nitrosoglutathione (GSNO).

In addition, investigating whether the phenotypes are affected by the stage of growth at which DNA damage occurs would be of interest, particularly for the NHEJ mutants. It has been suggested that NHEJ may be important for survival under conditions where the bacteria are not replicating, such as during latency and dormancy (Weller *et al.*, 2002)

In an effort to determine whether the altered susceptibility of the mutant strains to particular kinds of DNA damage impact on their ability to cause disease, it would be valuable to investigate the ability of these strains to survive and grow in additional models of infection, such as activated macrophage and whole animals.

Consequently, the NHEJ mutants (Δ Rv0937c, Δ Rv0938, Δ Rv0937c/ Rv0938) and the Rv2191 mutant have been chosen for further study to more thoroughly examine their phenotypes. It is hoped that more detailed investigation may deliver additional insight into the roles of these DNA repair pathways in Mtb.

4.6 Concluding remarks

The results from the disc diffusion assays showed that inactivation of each of the genes targeted had an effect on growth in the presence of one or more DNA damaging agents. This suggests that all of the proteins encoded by these genes are involved in some form of DNA repair. However, this was a basic screen and additional experiments would be valuable to confirm and extend these findings. There did not appear to be any major difference in growth in culture under standard (non-damaging conditions), or in growth *in vivo* (growth in macrophages), between the mutant and wild type strains. Despite some small differences in growth early in infection, these were all corrected by the end of the time course, suggesting that any differences are compensated.

5 Phenotype II: The role of Non-homologous End Joining in *M. tuberculosis*

Hypotheses:

- 1) NHEJ mutants display impaired DNA repair following exposure to DNA damage.
- 2) NHEJ is involved in survival of Mtb during infection.

5.1 Introduction

In chapter 4, the disc diffusion assay suggested a potential role for NHEJ in DNA repair *in vitro*, and subsequently the NHEJ mutants (Δ Rv0937c, Δ Rv0938 and Rv0937c/Rv0938 double mutant) were chosen for further study. It had previously been demonstrated that NHEJ plays a role in the repair of double strand breaks in Mtb *in vitro* (Weller *et al.*, 2002), and during the course of this study, a role in the repair of such lesions *in vivo* was reported in another mycobacterial species, *M. smegmatis* (Msm) (Gong *et al.*, 2005). The results obtained in the previous chapter were in agreement with these findings. However, there are as yet no reported *in vivo* studies concerning the effect of the inactivation of NHEJ on the survival and growth of Mtb in infection models such as macrophages or whole animals.

The disc diffusion assay showed that all of the NHEJ mutants exhibited increased sensitivity to bleomycin, a chemical inducer of DNA double strand breaks (DSB), and that the double mutant (Δ Rv0937c/Rv0938) displayed increased sensitivity to both mitomycin C, an alkylating agent, and menadione, which induces reactive

oxygen intermediates. Therefore, it was decided to further investigate the role of NHEJ in DNA repair in Mtb using a number of more probing experiments *in vitro* and investigating survival and growth *in vivo*.

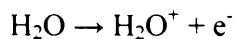
5.2 Exposure to Gamma Radiation

Ionizing radiation damages cellular components and is known to induce a variety of DNA lesions. The deposition of energy through ionizing radiation, leads to the formation of excited, ionized molecules, which can cause double strand breaks in the DNA via a number of distinct stages (Ward 1990).

- 1) Physical deposition of energy.
- 2) Production of primary radicals on the target molecule and in molecules surrounding the target. This is classed as both the direct and indirect effect. The direct effects occur as a result of the cellular DNA interacting with radiation energy. Conversely, the indirect effects occur as a result of the cellular DNA reacting with reactive species formed by the radiation.
- 3) Reaction of radicals on the surrounding molecules with the target molecule.
- 4) Reactions of unstable target radicals leading to chemically stable damage.

The outcome of the dose of radiation depends on the progression through the stages. If all of the stages remain constant under the dose given, the yield of the damage is linear with the dose.

The reactive species formed as a consequence of exposure to ionizing radiation are predominantly those formed by the radiolysis of water (Ward 1988):



Subsequent reactions form the hydroxyl radical ($\cdot\text{OH}$) and hydrogen peroxide. The hydroxyl radical is suggested to be the most damaging product of ionizing radiation for cellular DNA (Ward 1988).

5.2.1 Standardisation

The mutations induced by ionizing radiation cause double strand breaks in the DNA. For this reason it was decided to expose cultures to varying doses of gamma radiation from a Caesium source (^{137}Cs). The doses chosen were picked as they caused approximately 50% killing in the wild type strain. This would ensure that any hyper-susceptible mutants could be accurately identified. *Bacillus subtilis* NHEJ mutants show greatly increased susceptibility to ionizing radiation at doses as low as 50 greys (Weller et al 2002), so it was possible that a similar result would be seen for the Mtb NHEJ mutants. Following exposure to gamma radiation, the number of colony forming units (cfu), were determined as a measure of viability.

For the purposes of standardisation and optimisation of the experiment, the viability of the wild type bacteria was investigated at 2 time points following irradiation. Viability was measured following a 24 hour incubation period after exposure to gamma radiation (to allow repair to take place), and compared to cfu obtained from a sample of the same culture that was plated out immediately following exposure to gamma radiation (Figures 5.1 and 5.2).

Due to the drastic reduction in viability following immediate plating (Figure 5.1), it was decided to incubate the cultures in the main experiment for 24h following exposure to gamma radiation (Figure 5.2). This would allow the repair mechanisms to act and so should increase the probability of being able to detect a potentially hyper-susceptible mutant that is unable to perform the repair.

The link between the dose of IR and the quantity of double strand breaks induced is slightly unclear (Ward 1990), and it is unknown whether the effect is dose dependent. The amount of damage is dependent on the cell type and availability of oxygen (Ward 1990). As a rough guide, at doses of gamma radiation greater than 5 Gy, there are approximately 70 double strand breaks per Gy (Blöcher 1982). Using this correlation as a rough guide would quantify the dose used in the experiments here as follows:

$$50 \text{ Gy} = 3500 \text{ dsb per cell}$$

$$100\text{Gy} = 7000 \text{ dsb per cell}$$

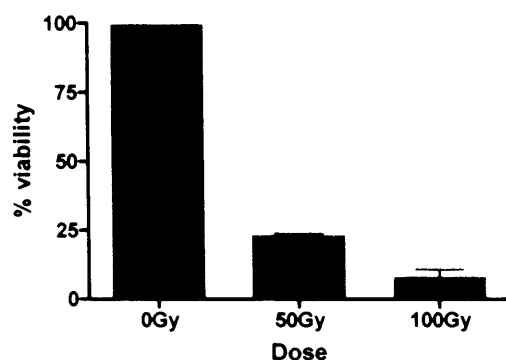


Figure 5.1: Standardisation experiment: Viability of the WT strain following exposure to Gamma radiation (no incubation stage).

Cultures were serially diluted and plated out immediately following exposure to radiation. Doses of 50 and 100 Gy were used, alongside an untreated control. After 2 weeks, cfu were counted and the results normalised by calculating viability as a percentage of the untreated value.

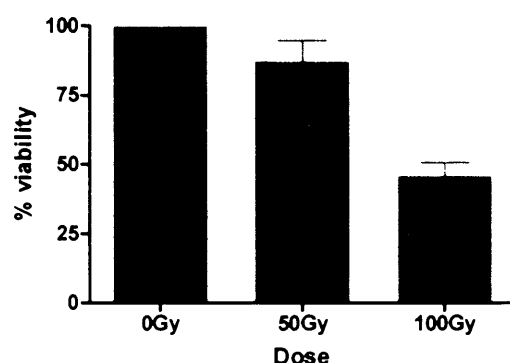


Figure 5.2: Standardisation experiment: Viability of the WT strain following exposure to Gamma radiation including incubation stage.

Cultures were incubated with rolling for 24 h following exposure to radiation. Doses of 50 and 100 Gy were used, alongside an untreated control. Following the incubation period, samples were serially diluted and plated out. After 2 weeks, cfu were counted and the result normalised by calculating viability as a percentage of the untreated value.

5.2.2 Gamma radiation Assay

Cultures were grown up to mid-exponential and stationary phase before being subjected to gamma irradiation from a Caesium source. After exposure, cultures were returned to rolling bottles and incubated for 24 h at 37°C with rolling prior to serial dilution and plating. Plates were incubated at 37°C for 2 weeks. Experiments were repeated with 3 biological replicates, unless stated otherwise, and plated in duplicate, for each strain.

5.2.3 Exponential phase

Cultures were grown up to an OD₆₀₀ of between 0.3 and 0.4 prior to exposure to the Caesium source. Perhaps surprisingly, the data obtained (Figure 5.3) revealed that although there appeared to be a slight reduction in survival for the double Rv0937c/Rv0938 mutant, there was no statistically significant difference between any of the NHEJ mutants and the wild type as determined by an unpaired T-test ($p > 0.05$). Overall, the results indicated that NHEJ is not essential in the repair of damaged DNA caused by exposure to these doses of ionizing radiation.

5.2.4 Stationary phase

As there was no significant difference in viability between the NHEJ mutants and wild type strain in exponential phase, despite the disc diffusion assay with bleomycin indicating an increased sensitivity to DNA double strand breaks, it was decided to investigate whether the NHEJ mutants in Mtb would be susceptible to ionizing radiation whilst in stationary phase. In this way, it would be possible to

gain clues to a potential role for these genes in repairing DNA damage during dormancy and latency, an important trait for survival *in vivo*. Cultures were grown in duplicate to an OD₆₀₀ of 2.0 and exposed to 50 and 100Gy as before, and viability compared with an untreated control. The results (Figure 5.4) show that there is no statistically significant difference in survival following gamma radiation between any of the mutants and wild type in stationary phase. There was also no difference observed between viability of the single mutants and the double mutant indicating that there is no difference if one member of the pathway is lost or if the entire pathway is eliminated. Again, the results suggest that for the repair of double strand breaks induced by these doses of Gamma radiation in stationary phase, NHEJ is not an essential repair mechanism. This may be due to the presence of multiple pathways for the repair of this kind of damage, reflecting its importance in Mtb.

Traditionally, it has been assumed that damage induced by ionizing radiation is repaired by HR, and this may well be the case here. It is possible that at higher doses, or later in stationary phase, a difference may have been detected but it is clear that removal of the NHEJ pathway in Mtb does not render the bacteria hyper-susceptible to the effects of ionizing radiation.

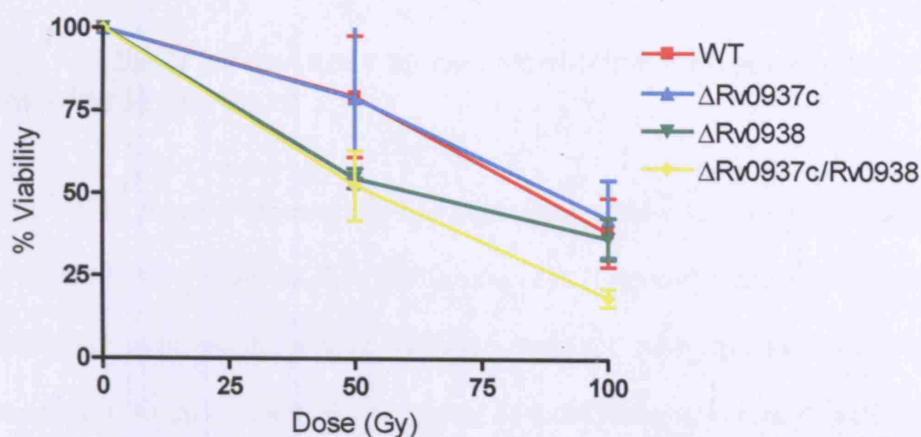


Figure 5.3: Susceptibility of mutants involved in NHEJ to gamma radiation during exponential phase

Mutant strains were grown up to an OD_{600} of 0.3-0.4 and subjected to 50 or 100Gy, or left untreated as a control. Cultures were returned to roller bottles and incubated for 24h at 37°C with rolling before being serially diluted and plated out. Data was normalised by expressing cfu at 50 and 100 Gys as a percentage of the mean value for the corresponding untreated controls. The graph shows a slight difference in response for the Rv0937c/Rv0938 mutant compared with the wild type strains but this is not statistically significant (Unpaired T test $p > 0.05$).

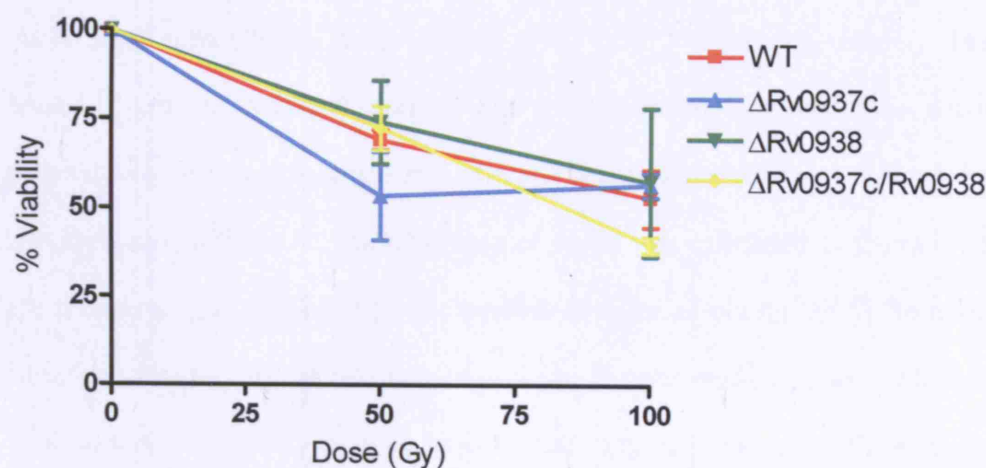


Figure 5.4: Susceptibility of mutants involved in NHEJ to gamma radiation during stationary phase

Mutant strains were grown up to an OD_{600} of 2.0 and subjected to 50 or 100Gy, or left untreated as a control. Cultures were returned to roller bottles and incubated for 24h at 37°C with rolling before being serially diluted and plated out. Data was normalised by expressing cfu at 50 and 100 Gys as a percentage of the mean value for the corresponding untreated controls. There is no statistically significant difference between the WT and mutant strains in susceptibility to gamma radiation during stationary phase (Unpaired T test $p > 0.05$).

5.3 Ability of mutants to re-circularise a linear plasmid: efficiency of repair

The NHEJ mutants showed an increased susceptibility to bleomycin compared with the wild type in the disc diffusion assay. However, a decrease in viability following exposure to gamma radiation was not observed. Therefore, it was decided to further investigate the ability of these mutants to repair double strand breaks. The assay used specifically tests the ability to repair double strand breaks via the ability to recircularise a linearised plasmid.

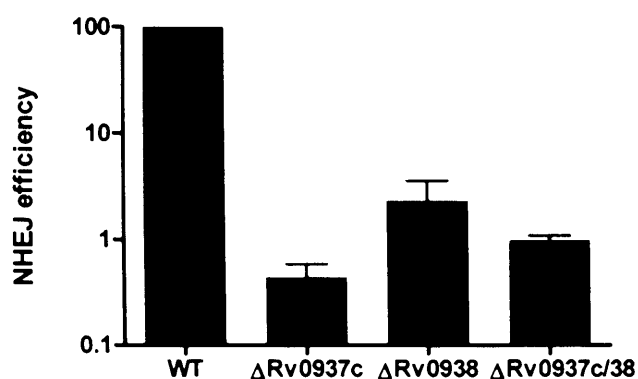
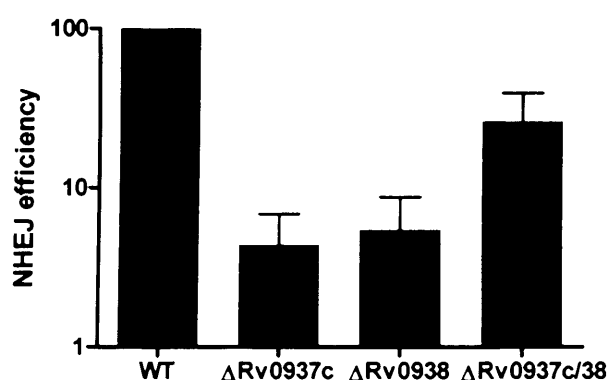
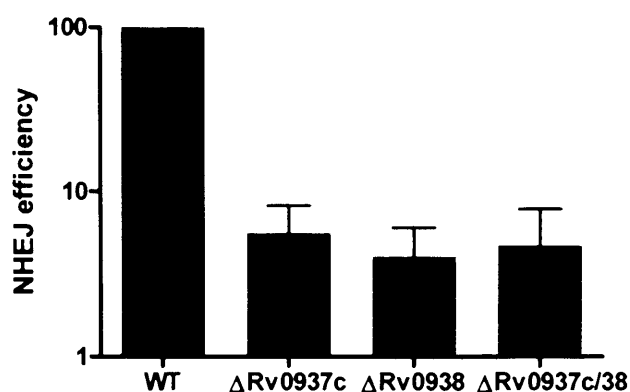
The plasmid used for this assay was pMV261 (Stover *et al.*, 1991), which is able to replicate in mycobacteria as well as in *E. coli* and confers kanamycin resistance. Plasmid DNA was linearised using a restriction enzyme which cut to leave blunt ends (*PvuII*), a 3' overhang (*PstI*) or a 5' overhang (*EcoRI*). The linearised plasmid was gel purified and electroporated into Mtb. The uncut plasmid was also electroporated into Mtb and acted as a control to determine the transformation efficiency. The efficiency of repair was calculated as the ratio of the transformation efficiency (ie the number of colonies per ng DNA) from the linearised plasmid to that resulting from uncut circular plasmid DNA. This was then normalised to the wild type ratio for the same type of cut to allow easier comparison (Gong *et al.*, 2005). A control transformation of *E. coli*, which lacks a NHEJ system, was also performed to confirm that the cut plasmids were fully linearised. Results demonstrated that less than 0.25% of the linearised plasmid remained uncut (data not shown).

An additional control experiment was also carried out to quantify the integration of linear plasmids into the Mtb chromosome. This was to ensure that the colony numbers obtained from the experiment were representative of repaired re-circularised plasmids and did not include any un-repaired, linear plasmids which may have inflated the result. This control was performed by electroporating a linearised non-replicating plasmid, pBackbone (Gopaul 2003), which also carried kanamycin resistance. The plasmid was cut where possible, with the same enzymes used for the main experiment (*PvuII* and *EcoRI*). This was not possible for the 3' overhang, as *PstI* is not a unique site in pBackbone. Therefore, *KpnI* was used instead to generate a 3' overhang. Uncut plasmid was also used as a control. The rate of random integration was calculated by comparing the number of colonies obtained from the control electroporations to the number of colonies resulting from the experimental electroporations. The results of this control experiment showed the rate of random integration to be negligible. It was therefore concluded that the rate of random integration would not affect the results of the primary experiment.

The NHEJ mutants all displayed an impaired ability to religate the blunt cut (*pvuII*) plasmid (Figure 5.5a), with the efficiencies generally between 40- and 100-fold less than in the wild type for the Rv0938 and Rv0937c/Rv0938 mutant strains. Repair efficiency of the Rv0937c mutant strain decreased approximately 200 fold compared to the wild type strain. The repair efficiency of the mutant strains were all statistically significantly different from that of the wild type (ANOVA $p < 0.0001$), but not from each other (ANOVA $p > 0.05$).

The repair efficiency for mutants repairing the plasmid cut with *PstI* to leave a 3' overhang showed a significant difference (ANOVA $p < 0.0001$) between the 3 NHEJ mutant strains and the wild type (Figure 5.5b). The Rv0937c and Rv0938 mutant strains had the lowest efficiency at around 20 to 30 fold less than the wild type, while although the double mutant appeared to be reduced to a lesser extent, its repair efficiency was not statistically significantly different from the single mutants (ANOVA $p > 0.05$).

The repair efficiencies of the mutants cut with *EcoRI*, to leave a 5' overhang, each showed a decrease of around 20 fold (Figure 5.5c). Again the values obtained for the mutant strains were all statistically significantly different from the wild type (ANOVA $p < 0.001$) but not from each other ($p > 0.05$).

a) Repair efficiencies of mutants cut with *PvuII* (blunt)b) Repair efficiencies of mutants cut with *PstI* (3' overhang)c) Repair efficiencies of mutants cut with *EcoRI* (5' overhang)**Figure 5.5: Repair efficiency of NHEJ mutants to re-circularise a linearised plasmid**

Mtb competent cells were transformed with circular or linearised plasmid (pMV261). Double strand breaks were made using restriction enzymes to leave blunt, 3' or 5' overhangs. The efficiency ratio was calculated as the number of Km^R colonies resulting from the transformation with cut plasmid, divided by the number of Km^R colonies resulting from the transformation with the uncut plasmid. This was then normalised to the value for the wild type. Statistical analyses between strains were carried out using a One-way ANOVA followed by Tukey's multiple comparison test. All strains were significantly different from the wild type but not from each other.

The decrease in ability to recircularise a linearised plasmid supports a role for Rv0937c and Rv0938 in double strand break repair and further implicates NHEJ as an important pathway for DNA repair in Mtb, at least in the absence of regions of homology.

Published studies have focused on the single mutants only and are restricted to Msm. These studies have investigated the ability to repair blunt and 5' ends only. The results obtained in this study for the single mutants for the repair of 5' and blunt ends are generally consistent with, and therefore supported by published results (Gong *et al.*, 2005; Akey *et al.*, 2006). The result obtained here gives a clear indication of the importance of NHEJ in DSB repair and hints at the role it plays.

5.4 Growth *in vivo*

The increased susceptibility to bleomycin and the decreased ability to re-circularise a linearised plasmid in the Rv0937c and Rv0938 mutants confirms a role for NHEJ in the repair of DSBs in Mtb. This raises the question of the potential importance of the system during infection.

Mtb is an intracellular pathogen and is therefore exposed to DNA damaging conditions during infection *in vivo*. In order to fully investigate the role of these genes in survival in an infection model, where the conditions are expected to create DNA damage, it was decided to look at growth of the mutants in activated macrophages. In addition, the ability of the different strains to grow and persist in a mouse model of infection were examined to investigate any changes in virulence of the mutant strains compared with the wild type strain.

5.4.1 Growth in activated macrophages

Growth *in vivo* was previously investigated using unactivated macrophages. Although it can be argued that one of the traits important for Mtb survival *in vivo* is the ability to survive in macrophages whilst preventing phagolysosome fusion (Armstrong and Hart 1975), activation of macrophages by cytokines, induced as part of the adaptive immune response can overcome this block and promote intracellular killing (Vergne *et al.*, 2004). Furthermore, mutants deficient in various components of DNA repair pathways show impaired survival *in vivo*, demonstrating the damaging effects of the macrophage environment (Darwin and Nathan 2005). Inside the macrophage, it is probable that bacteria must withstand

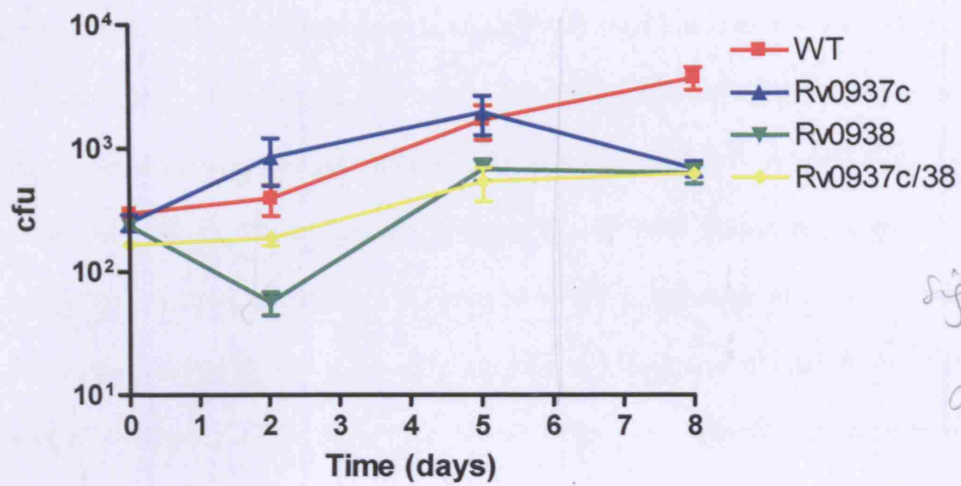
some level of activation in order to survive, and during the quiescent phase of infection, it is likely that the bacteria have to withstand low level assault from damaging agents. For these reasons, it was decided to investigate survival of the NHEJ mutant strains in an activated macrophage model of infection.

To investigate the growth of the mutants in macrophages, bone marrow derived murine macrophages from Balb/c mice were used. Primary macrophages provide a relatively lifelike model of infection, however, variability is greater compared with a cell line. The optimum test for bacterial survival of Mtb in macrophages would be using human alveolar primary macrophages, where bacterial survival in early infection can be investigated in their natural host cells. These cells are, for obvious reasons, hard to obtain. There are human macrophage cell lines available, such as U937 (Sibille *et al.*, 1994), which can be used for investigating mutant survival but only macrophages from TB infected individuals actually kill Mtb (Smith 2003).

Murine macrophages can be activated by the addition of IFN- γ and / or bacterial lipopolysaccharide which lead to the upregulation and production of the iNOS enzyme which catalyses the reaction to form NO (Chan *et al.*, 1992). *In vivo*, macrophages are activated predominantly by IFN- γ , augmented by IL-2 production from T helper (CD4) cells. Activated macrophages can inhibit growth and kill Mtb. Therefore measuring growth in activated macrophages is a means of testing whether the mutants, deficient in components involved in the repair of damaged DNA, will show attenuation under damaging conditions *in vivo*.

In this study, macrophages were activated by the addition of IFN- γ and bacterial LPS 24 h prior to infection with the mutant strains to ensure activation. The level of activation was confirmed using a Griess assay. The activated macrophages were shown to produce NO at approximately 30 μ M, a level consistent with that of activated macrophages (Darwin and Nathan 2005).

Although the NHEJ mutants showed variable growth at early time points after infection of activated macrophages, they were all attenuated compared to the wild type strain at day 8 (Figure 5.6). This difference at day 8 was statistically significant (Unpaired T-test $p < 0.0001$). Interestingly, there was little variation between the mutant strains at this timepoint, suggesting that the loss of any component of the pathway has a detrimental effect on survival at later timepoints during infection in macrophages. This may have an interesting bearing on virulence in an animal model.



significance of
day 8?

Figure 5.6: Growth of NHEJ mutants in activated macrophages

Macrophages were activated by the addition of IFN- γ (100u/ml) and LPS (1 μ g/ml) 24h prior to infection with Mtb. Experiments were performed with 3 biological and 2 technical replicates.

All NHEJ mutant strains show a statistically significant attenuation in growth compared to the wild type strain at day 8 (Unpaired T test $p < 0.0001$).

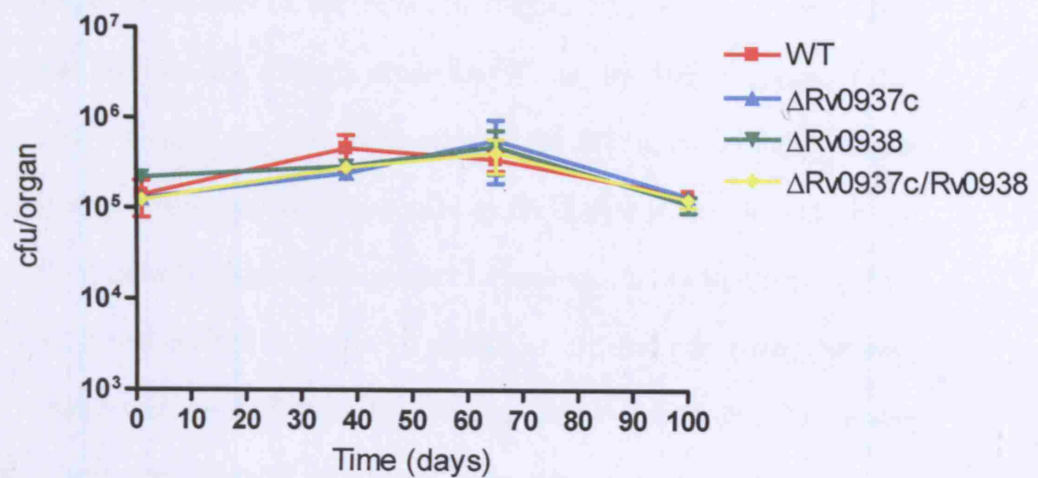
5.4.2 Growth in mice

Infection of isolated macrophages is an artificial situation that is much simpler than infection of a whole animal. Therefore, the results of such studies are not always indicative of phenotypes to be found in an animal model, where many other factors come into effect. These include components of both innate and adaptive host immunity. In order to confirm the role of NHEJ in survival during infection, it is necessary to examine survival of the mutants in a complete animal model. For this reason, the ability of the strains to cause progressive infection in mice was also compared.

Growth of the NHEJ mutants was assessed *in vivo* using a mouse intravenous infection model. Five mice from each group, infected with a specific mutant or wild type strain, were sacrificed at each time point (1, 38, 65, 100 days) and cfu from lung and spleen were determined by homogenizing tissue and plating out serial dilutions.

All of the NHEJ mutants, $\Delta Rv0937c$, $\Delta Rv0938$, $\Delta Rv0937c/Rv0938$, displayed the same pattern of growth in the spleen as for the wild type strain (Figure 5.7a). Spleen weights of the sacrificed animals infected with the mutant strains from each time point were compared with those for animals infected with the wild type strain (Figure 5.7b). There was a statistically significant difference in weight between the $Rv0937c$ mutant and wild type strain (Unpaired T test $p=0.0046$).

a) Growth of NHEJ mutant strains in mouse spleens



b) Weight of mouse spleens infected with NHEJ mutant strains

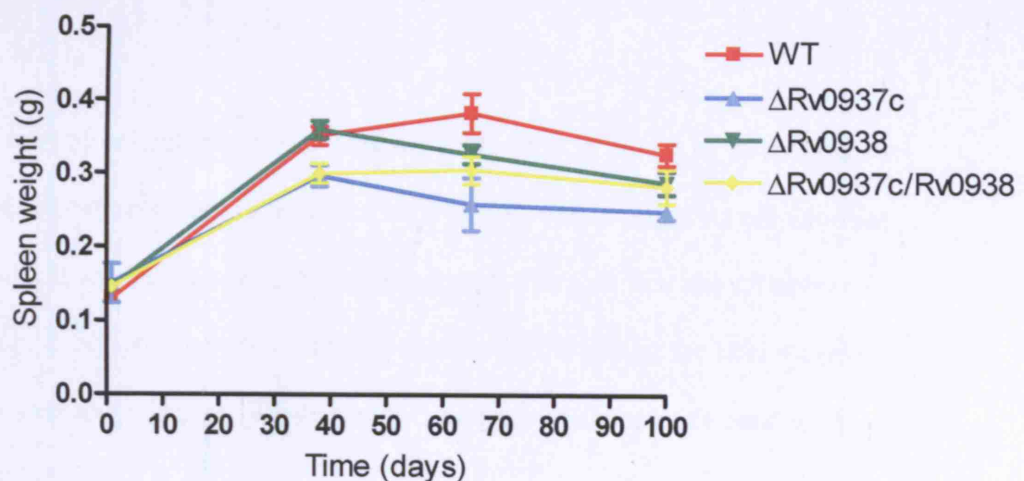


Figure 5.7: Growth of the NHEJ mutant strains in mouse spleens

Five mice infected with each strain were sacrificed at each time point, and their spleens homogenized, serially diluted and plated out (part a). The growth of the mutant strains is similar to that of the wild type. Prior to homogenization, the spleens were weighed (part b). There is a significant difference between the weight of the WT infected spleen, and that of the $\Delta Rv0937c$ infected spleen (Unpaired T test $p = 0.0046$).

when?

In the lungs, the pattern of growth for each of the mutants was similar to that of the wild type strain early in the infection (Figure 5.8). All the of the NHEJ mutants are significantly different from the WT at day 100 (Unpaired T test $p < 0.005$). However, as the inoculating dose of the WT strain is slightly higher than the mutant strains, and is phenocopied by the double mutant, it is likely that the pattern of growth of the double mutant follows that of the wild type, and for this reason, it was decided to confirm a significant difference in growth between single mutants and the wild type, by comparing their growth with the double mutant. There was a statistically significant difference in growth observed between both single mutants and the double mutant strain (Unpaired T test $p < 0.004$).

In order to explore this potential phenotype more fully, it would be interesting to repeat the experiment over an extended time period, which might reveal a further decline in cfu as the time of infection progresses. The fact that the difference is seen late in infection points to a possible role for NHEJ during the later stages of infection, perhaps in latency. This could be a very interesting area to pursue.

It was surprising that the Rv0937c/ Rv0938 double mutant, lacking the entire NHEJ pathway appeared to be less affected in the mouse model than the single Rv0937c mutant. It is possible that removal of the NHEJ pathway facilitates the activity of another repair mechanism, which is hindered by components remaining in the single mutants.

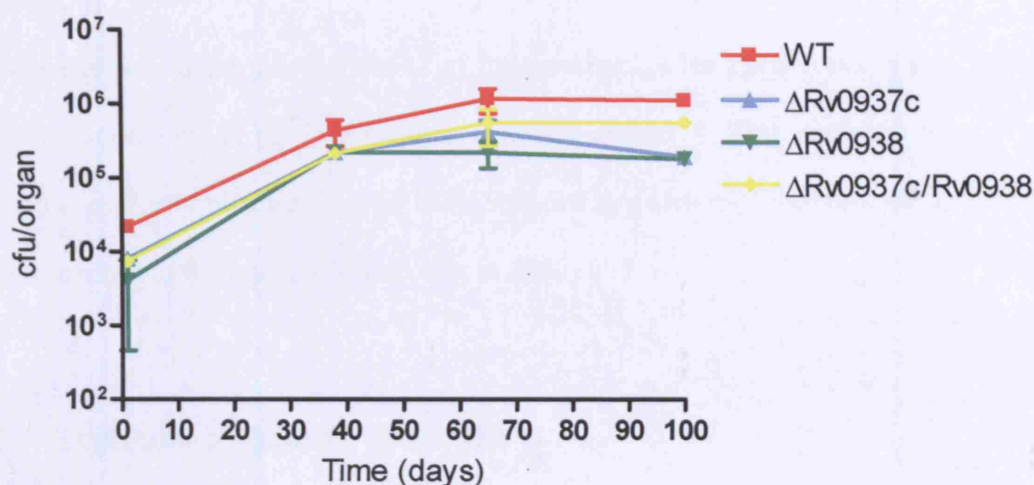


Figure 5.8: Growth of the NHEJ mutant strains in mouse lungs

Five mice infected with each strain were sacrificed at each time point, and their lungs homogenized, serially diluted and plated out. The growth of the mutant strains is similar to that of the wild type during early infection.

There is a significant difference between growth of the WT and all NHEJ mutant strains at 100d, as determined by an Unpaired T test:

$\Delta Rv0937c$ $p=0.0001$

$\Delta Rv0938$ $p=0.0001$

$\Delta Rv0937c/Rv0938$ $p=0.005$

In order to confirm this difference and ensure that it was not due to the higher starting inoculum of the wild type strain, it was assumed that the $Rv0937c/Rv0938$ mutant phenocopies growth of the WT strain. Statistical analyses of comparison of the single mutants to the double mutants confirmed a difference between $\Delta Rv0937c$ and $\Delta Rv0937c/Rv0938$ (Unpaired T test $p=0.004$) and between $\Delta Rv0938$ and $\Delta Rv0937c/Rv0938$ (Unpaired T test $p=0.0036$).

5.5 Discussion

The experiments in this chapter aimed to further examine the phenotypes of the NHEJ mutants, expanding on the observations in chapter 4. This investigation focused specifically on the effects of DSBs induced in a variety of different ways, and on survival of the mutants in infection models.

5.5.1 Exposure to gamma radiation

Exposure to gamma radiation was shown not to affect the viability of the mutant strains any more than the wild type at the doses used in either exponential or stationary phase. In view of the increased sensitivity to bleomycin observed in Chapter 4, it was surprising that none of the NHEJ mutants exhibited enhanced sensitivity to killing by gamma radiation. The doses used here were similar to those used previously to demonstrate increased sensitivity to ionizing radiation in NHEJ mutants of *Bacillus subtilis* (Weller *et al.*, 2002). If anything, the survival of the wild type Mtb strain was lower than that reported for wild type *B. subtilis*, suggesting that sufficient DNA damage was incurred. This observation is supported by recent work on *B. subtilis* NHEJ mutants, where differences in viability between mutant and wild type strains were apparent at low doses of radiation (Moeller *et al.*, 2007). Additionally, a dose of 100 Gy was sufficient to demonstrate a difference in viability between the *M. smegmatis* wild type and a *uvrD1* mutant strain (Sinha *et al.*, 2007). *uvrD1* is a component in an alternative DNA repair pathway, nucleotide excision repair, and was shown in that study to play a role in NHEJ as well (Sinha *et al.*, 2007).

Therefore, it was expected that any difference in sensitivity amongst the strains should have been detectable in the experiments presented here. It is possible, that with an increased dose of gamma radiation, a difference might become apparent. A recent paper on the effects of ionizing radiation on NHEJ mutants in *M. smegmatis*, showed that at high doses of radiation, the *ku*, *lig* and *ku/lig* mutants were more significantly affected compared with the wild type strain (Pitcher *et al.*, 2007). This is interesting as the mutant strains were only more sensitive in stationary phase and the sensitivity observed was not as marked as that demonstrated in *B. subtilis* (Weller *et al.*, 2002). Another recent paper demonstrated that the loss of components of the NHEJ pathway, sensitizes Msm to high doses of ionizing radiation only very late in stationary phase and only in minimal media. This may support a role for the pathway in repair during latency. In this study, the single mutants were more affected than the double mutant (Stephanou *et al.*, 2007).

The discrepancy between the results for Msm NHEJ mutants and those obtained here for Mtb NHEJ mutants may be explained by the fact that the doses of ionizing radiation used were significantly higher for Msm. Due to limitations with the radiation source used, and safety considerations associated with working with Mtb, it was not possible to use such high doses in this study. Additionally, as the dose used in this study was given over a prolonged period of time, due to the only available source of gamma radiation being comparatively weak, it is possible that the bacteria were able to repair the damage during the exposure period and were less affected by the dose.

It remains a possibility that Msm and Mtb mutants differ in their response to gamma radiation and in their repair of double strand breaks. This would not be altogether surprising as there are some significant differences between Mtb and Msm, not least the fact that Mtb is a pathogen whilst Msm is not. Furthermore, the chromosomal location of the genes differs between Mtb and Msm. In Mtb, Rv0937c and Rv0938 are divergently transcribed, whereas in Msm, the genes are not linked. It is probable that if DNA repair is a factor in survival of the bacteria *in vivo*, there may be additional repair mechanisms in Mtb, which contribute to its survival in the absence of one repair pathway. Msm does not have to withstand such damaging effects of the host immune response in order to survive, as it is not a pathogen under normal circumstances, and therefore may not be so well adapted to survival under these specific DNA damaging conditions.

Further investigations could include examining viability following exposure to gamma radiation in the Wayne model of dormancy (Wayne and Hayes 1996). It would also be interesting to investigate viability later in stationary phase, which is more representative of conditions during the quiescent phase of survival in Mtb infection *in vivo*, where bacterial replication is reduced. The mutants under investigation here could be combined with mutations in other DNA repair genes thought to play a role in the repair of double strand breaks, for example *recA*.

It must be noted that Mtb would never normally be exposed to gamma radiation *in vivo*, this is merely used as a tool to model the effects of double strand breaks. However, it has been reported that there is a link between the ability to withstand ionizing radiation and the ability to withstand desiccation (Sanders and Maxcy

1979). As desiccation induces the formation of double strand breaks and is an important barrier to effective transmission of Mtb from host to host, it would be interesting to investigate this and observe whether the NHEJ pathway is involved in repairing damage incurred as a result of exposure to desiccation. NHEJ in Msm has been implicated in survival against desiccation (Pitcher *et al.*, 2007). This may have a bearing on the successful spread of Mtb but equally, may differ between Msm and Mtb. As Msm is an environmental pathogen, it is likely that it must withstand higher levels of desiccation compared with Mtb. The importance of NHEJ in repairing damage resulting from desiccation may be supported by the fact that spores of *B. subtilis* NHEJ mutants are more sensitive to desiccation compared with those of WT strains, and this presumably has a bearing on their ability to survive in the environment for extended periods of time (Moeller *et al.*, 2007). It therefore seems likely that survival against desiccation and, linked with this, survival against gamma radiation are more important attributes for environmental bacteria than disease causing human pathogens such as Mtb. However, the fact that Mtb is less susceptible to gamma radiation may be related to the fact that its repair pathways are better adapted to repairing DSBs in order to ensure survival *in vivo* during infection, and as a consequence is well adapted to repair of damage sustained from ionizing radiation.

5.5.2 Efficiency of Repair

Efficiency of DNA repair was investigated by the assay quantifying the ability of the mutant strains to re-circularise a linearised plasmid. All three NHEJ mutant strains exhibited a reduced ability to repair double strand breaks in this assay.

The Rv0937c mutant was found to be slightly more deficient in the repair of all 3 types of double strand break examined compared with the other NHEJ mutant strains. Efficiency of repair was severely impaired and reduced approximately 200-fold compared with the wild type for repairing blunt ends, and approximately 20-fold for repairing 5' and 3' ends. This decrease in the efficiency of the repair of blunt ends in Mtb was similar to that detected in Msm by Gong *et al* (2005). For the repair of 5' ends, the level of repair observed here was higher compared with that demonstrated for Msm (Gong *et al.*, 2005). This may be due to differences between the functions of Rv0937c and Msm *ku* but still reflects a significant decrease in efficiency compared with the wild type strain and supports the results for Msm. Rv0937c acts by stimulating the ligation of compatible complementary ends by Rv0938 (Weller *et al.*, 2002) and so far, an alternative gene, able to act with the ATP-dependent ligase component, has not been identified. This may explain why this mutant was the most significantly impaired.

The Rv0938 mutant strain displayed a 20- to 50- fold decrease in efficiency of repair for all 3 types of overhang. The results showed that the Rv0938 mutant was better able to repair a double strand break compared with the Rv0937c mutant, most noticeably where the break had blunt ends. The Rv0938 homologue in Msm, *ligD*, is responsible for fill-in insertions during the repair of 5' cut DSBs, which supports a role for the polymerase domain of LigD in the generation of mutants (Gong *et al.*, 2005). In Msm, repair of 5' ends was decreased 20- fold (Gong *et al.*, 2005); a similar decrease in efficiency was demonstrated in this study. For the repair of blunt overhangs, efficiency fell to 2%. A similar figure was obtained for the repair of blunt cuts in Msm (Gong *et al.*, 2005). It has been demonstrated in

Msm that in the absence of LigD, Ku can act with LigC, an alternative ATP-dependent ligase. This pathway is Ku-dependent and the efficiency of repair of blunt ends is decreased 20-fold in a *ligC/ligD* mutant compared with the *ligD* mutant on its own (Gong *et al.*, 2005). It is possible that the finding that the Rv0938 mutant is less affected than the Rv0937c mutant is due to an interaction between Ku and LigC, as is suggested to be the case in Msm (Gong *et al.*, 2005). A homologue to *ligC*, Rv3731, is present in Mtb.

The Rv0937c/Rv0938 mutant displayed a 4-fold reduction in ability to repair 3' overhangs and a 20-fold reduction in ability to repair 5' cuts. A marked decrease was observed in repairing blunt ends as shown by a decrease of around 100-fold compared to the wild type. These results support a role for NHEJ, particularly in the repair of blunt ends. Although the single NHEJ mutants appeared slightly less able to repair the damage compared with the double mutant, the difference between the strains was not statistically significant.

The results obtained from the disc diffusion assay screen (chapter 4) showed the growth of the single mutants to be less affected following exposure to some DNA damaging agents. However, it is difficult to compare the damage incurred in the disc assay to the complete breakage following incubation with restriction enzymes. Almost 100% of the plasmid DNA was linearised in the religation assay, which implies that re-circularisation depends on the repair pathways religating the introduced DNA. The overhangs made by incubating the plasmid with restriction enzymes are fully compatible and complementary. This can have a bearing on the ability of the components of NHEJ to repair the damage (Della *et*

al., 2004). At incompatible ends of long linear DNA molecules, the action of Rv0937c is slightly different; it stimulates the ligation by Rv0938 but does not have a significant effect on the removal of mismatched flaps. Instead, it prevents digestion of the microhomology region (Della *et al.*, 2004). Presumably the ends made by DNA damaging agents *in vitro* or *in vivo* are not complementary and require significant processing by nucleases and polymerases prior to ligation (Della *et al.*, 2004). This could explain the discrepancy between the re-ligation assay and some of the *in vitro* susceptibility experiments, although the difference between strains in this re-ligation assay was not statistically significant.

It is possible that once the NHEJ pathway is completely removed, other pathways are able to compensate its lack. The results of the re-circularisation assay support the importance of NHEJ in the repair of DSBs but do not give an idea of its use *in vivo* as DNA damage *in vivo* would never be as extreme. A recent study has demonstrated that repair function in the double Ku/Ligase mutant is less impaired in Msm and does not phenocopy the single mutants (Stephanou *et al.*, 2007). It is hypothesized that presence of Ku or Ligase may inhibit other repair proteins or pathways, which act when the NHEJ pathway is inactivated. This may suggest functional interactions between DNA repair pathways (Stephanou *et al.*, 2007), although recent work has suggested that RecA is not important for NHEJ in Mtb (Malyarchuk *et al.*, 2007).

The results still give a clear indication of the importance of NHEJ in DSB repair. Although this has been demonstrated in Msm (Gong *et al.*, 2005), along with the findings that the pathway is very inefficient and mutagenic (Gong *et al.*, 2005;

Akey *et al.*, 2006), this is the first time it has been shown to be such an important component of this kind of repair in Mtb.

It has been demonstrated that the expression of Rv0937c and Rv0938 in *E. coli* resulted in inaccurate repair and the deletion of sequences in a plasmid re-joining assay (Malyarchuk *et al.*, 2007). It would be interesting to examine the sequence of the re-joined plasmids from the experiment presented here, and therefore further investigations could include looking at the fidelity of the repair process. The question of pathway fidelity is an interesting one. Results from Msm (Gong *et al.*, 2005) showed the pathway to have a very low fidelity, which leads to the question of whether this may be an important factor in the survival of Mtb *in vivo*.

The importance of the Pol domain in Msm LigD in the induction of error prone mutation during repair has been demonstrated, (Zhu *et al.*, 2006) and this polymerase activity is present in Mtb Rv0938 (Della *et al.*, 2004). Mutations induced by oxidative or nitrosative damage *in vivo*, or by chemotherapy to treat Mtb may encourage production of more resistant generations of bacteria in this way by inducing mutations which are not accurately repaired. Additionally, it would be interesting to investigate the potential role of NHEJ in selecting for antibiotic resistance. *DnaE2*, a DNA damage inducible gene which encodes the DnaE2 protein, an error prone DNA polymerase, is important in the emergence of drug resistance *in vivo* (Boshoff *et al.*, 2003). This supports the role of error prone repair or mutation inducing repair in survival in the presence of antibiotics via the production of resistance, and may imply that repair, even if inaccurate, is preferable to cell death and may even confer an evolutionary survival advantage.

Mutating other ligases would investigate whether the decreased repair observed in the NHEJmutants is affected by the presence or absence of LigC. LigC appears to be involved in NHEJ in the absence of LigD, equivalent to Rv0938, in Msm (Gong *et al.*, 2005).

5.5.3 Growth *in vivo*

Growth *in vivo* is thought to be, at least in part, dependent on the bacteria being able to repair damage caused by reactive oxygen and nitrogen intermediates, generated by the host immune response. If the genes under investigation are involved in DNA repair, the phenotype of the mutants *in vivo* might be attenuated. However, there are a number of pathways for repairing damaged DNA and if one is removed or disabled, there may be others that can replace it.

Although there were no differences in growth observed for the mutants *in vitro*, and in unactivated macrophages (Chapter 4), a statistically significant difference was seen in growth for all of the NHEJ mutants in activated macrophages. A statistically significant decrease in bacterial load was observed in the Rv0937c and Rv0938 mutants at day 100 in mouse lungs. This was supported by a decrease in spleen weight for the Rv0937c mutant strain. Although there was also a significant difference recorded between the double mutant and the wild type, this was probably due to the fact that the wild type strain started with a higher inoculated dose. The results obtained here suggest a potential role for the NHEJ pathway of DNA repair late in infection and may again suggest that loss of the Ku or Ligase components of the pathway is more damaging than when they are inactivated together. This is supported by recent work (Stephanou *et al.*, 2007)

and could be further investigated by screening for protein interactions to identify potential binding partners.

If it were possible, it would be interesting to carry out a 'time to death' experiment to compare growth of the mutant and wild type strains in an animal model. This could be measured as time to a defined point of severe disease, such as a specified decrease in body weight, and would allow investigation into the effect of the mutated gene during the later stages of infection. The results obtained in this study would need to be confirmed by complementation to demonstrate that the growth phenotype observed is due to the effect of the mutation of the gene itself and not a mutation elsewhere in the chromosome.

Further experiments with macrophages could include looking at the survival of the NHEJ mutants in *phox*⁻ macrophages. This would remove the oxidative stress response and the products of ROI. If the mutants are attenuated *in vivo* due to the consequences of exposure to oxidative damage, this should revert back to the WT level in *phox*⁻ macrophages. This would then confirm the role of NHEJ in survival following damage induced by ROI specifically.

A disadvantage of using the mouse model is that some aspects of persistence and latency are not well modelled, and this is a stage of infection where DNA repair mechanisms may be important. However, it is thought that progression of infection is controlled to some extent by the mouse immune system as immunosuppression during the plateau phase of growth *in vivo* leads to bacterial replication and evidence suggests that the bacteria do not replicate during this

phase of infection in mice (Muñoz-Elías *et al.*, 2005). However, progression of infection in mice is undoubtedly different to that in humans.

The use of guinea pigs and rabbits as alternative animal models has been suggested, and rabbits show the same stages of disease as humans which is an advantage. Infection kinetics in mice, guinea pigs and rabbits are all similar, but a disadvantage of using guinea pigs and rabbits lies in the handling and space requirements. However, rabbits can develop granulomas and caseous lesions as in human infection (Converse *et al.*, 1996) which allows disease progression and virulence of mutant strains to be more accurately assessed. The effect of the strain on the host immune response can also, therefore, be more accurately investigated.

Most strains of mice are susceptible to Mtb infection and all mice eventually succumb to infection with wild type Mtb, but in humans only around 10% of healthy individuals develop the disease. Bacterial load in mice is much higher during persistent infection (Manca *et al.*, 2001). Additionally, mice do not develop the necrotic lesions which are seen in humans with advanced disease pathology, unless they are deficient in IFN- γ , NOS2 or TNF- α . Another limitation of using mice as an animal model of Mtb infection is their size. Their lungs and therefore alveoli and air sacs are small and not particularly comparable to a human lung model.

For investigations into the immune response, the mouse model may not be the most suitable but despite the associated limitations, it does allow us to screen our mutants and investigate their role in infection and whether they have a phenotype

in vivo. For the purpose of further investigating the results obtained in this study, it may be interesting to look at growth of the mutant strains in an animal model which more closely models conditions of latent infection within a granuloma.

A major limitation of the animal model used here was the fact that mice were injected with Mtb through the tail vein and not infected using an aerosol system. In natural infection, TB is almost always transmitted by inhalation and reaches the lungs where it can survive and replicate in macrophages. Interestingly though, Mtb can cause infection in virtually all organ systems. When administered intravenously, the infection progression will be different. This route of infection also precludes the identification of genes required for dissemination from the lung, although this would be an unlikely phenotype for genes involved in DNA repair. However, for the simple screening of mutants for phenotypes, the intravenous infection model should still give an idea of whether the genes under investigation are involved in virulence although the pathology may be slightly different.

It has been reported that in some cases, phenotypes apparent in one animal model can be slightly different or even completely reversed in another model (Sun *et al.*, 2004). This implies that for confirming a definitive mutant phenotype, a number of animal models would give a more accurate result and also that the different immunological make up of different species may affect the outcome of infection.

5.5.4 Future work

The next stage in this work is undoubtedly to construct a complemented strain and assess whether the phenotypes observed in the mutant are restored to those of the wild type. This would be particularly beneficial to confirm the phenotype observed in the activated macrophage model and could be followed by further growth and viability experiments. Growth in phox- macrophages and additionally reactivation following growth in an *in vitro* model of dormancy would be useful in investigating whether this pathway is important for survival at later stages in the progression of infection. It may also be interesting to investigate growth of the NHEJ mutants over a longer period of time in mice to observe whether the genes may be involved in survival later on in infection progression, a possibility hinted at by the data obtained here. This could be investigated using a 'time to death' experiment and a more sophisticated animal model. Further characterisation of the mutants could be performed to investigate viability following exposure to DNA damaging agents, and the effect of such exposure on the expression of genes belonging to alternative repair systems could be investigated using microarrays.

5.6 Concluding Remarks

The different tests to determine viability and the ability to repair DNA double strand breaks clearly show a role for all the genes under study, and therefore NHEJ, in the repair of DNA damage. The mutants show impaired DNA repair or decreased growth under certain DNA damaging conditions, both *in vitro* and *in vivo*. The increased sensitivity of the Rv0937c/Rv0938 mutant to menadione,

which mimics conditions of oxidative stress, and of all the NHEJ mutants to bleomycin, which causes double strand breaks, confirms a role for the Mtb NHEJ pathway in the repair of damage. The drastic impairment in ability of the NHEJ mutants to religate blunt, 3' and 5' overhangs further highlights the importance of the pathway in this kind of repair.

The decreased ability of the single mutants to repair a linearised plasmid and their slightly attenuated growth in mice compared with the double mutant strain may suggest that the single remaining component interacts with or disrupts alternative repair pathways in some way.

Attenuated growth of the strains in activated macrophages leads to speculation that *in vivo*, the NHEJ pathway is important in the repair of damage sustained as a result of exposure to ROIs inducing DSB in the bacterial DNA. This potentially hints at an important role for NHEJ in survival *in vivo*. If the fidelity of NHEJ in Mtb is as low as it has been reported to be for Msm (Gong *et al.*, 2005), it is tempting to speculate that NHEJ is not only important for survival during infection as suggested here, but may also play a role in the evolution of antibiotic resistance, a function also demonstrated in the case of a different DNA repair gene (Boshoff *et al.*, 2003).

In conclusion, NHEJ mutants do display an impairment in DNA repair following exposure to DNA damage and the data presented here does support an involvement for NHEJ in the survival of Mtb during infection *in vivo*, as demonstrated in a mouse model.

6 Phenotype III: The role of Rv2191 in survival following DNA damage

Hypotheses:

- 1) The Rv2191 mutant is susceptible to DNA damage *in vitro*.
- 2) The Rv2191 mutant is affected in its ability to grow *in vivo*.

6.1 Introduction

In this chapter, the hypothesis that the Rv2191 mutant is particularly susceptible to DNA damage will be thoroughly investigated by determination of bacterial survival following exposure to DNA damaging chemicals. The hypothesis that this mutant is affected in its ability to grow *in vivo* will be assessed by quantification of bacterial viability following infection. In both cases, it will be confirmed that any phenotypes observed are due to mutation of Rv2191 by use of a complemented strain.

Following on from the finding that the Rv2191 mutant was particularly susceptible to menadione in a disc diffusion susceptibility assay, the potential sensitivity of this mutant to oxidative damage will be fully investigated in this chapter. This will be undertaken using a range of DNA damaging compounds, known to induce Reactive Oxygen Intermediates (ROIs). The effect of exposure to nitrosative damage will also be investigated, to model the effects of damage incurred in macrophages as a result of exposure to the host immune response. The fact that the mutant was also susceptible to ofloxacin, which is thought to induce

damage in the form of bulky adducts, supports a role for Rv2191 in the repair of nitrosative damage.

The second part of this chapter will investigate survival of the mutant in activated macrophages and mice. This will give a clue as to whether the Rv2191 gene, and potentially the Nucleotide Excision Repair pathway, is involved in survival *in vivo*, and thus whether the repair mechanism is implicated in the repair of damage sustained by exposure to the host immune system. Results from an animal model of infection can be used to further confirm or query a potential phenotype, and are key to assessing any role in pathogenesis.

6.2 Complementation

In order to confirm that any potential phenotype is the result of the missing gene, a complementing plasmid must be constructed, whereby the gene is reintroduced into the mutant strain. The phenotype should be reverted back to wild type in the complemented strain and this confirms that the phenotype observed is due to the effect of the mutation introduced in that particular gene.

The complementing plasmid was constructed by cloning the gene fragment into a TOPO vector (Invitrogen) and subsequently cut out using restriction enzymes, and transformed into a non-integrating suicide plasmid, pKP186 (Rickman *et al.*, 2005). Potential constructs were screened using restriction digests at various stages and the identity of the complementing construct was confirmed by sequencing prior to electroporation into the mutant strain. The complementing

plasmid was electroporated into $\Delta Rv2191$ competent cells along with a suicide plasmid (pBS-int) expressing *integrase* (Springer *et al.*, 2001).

Following electroporation, the complementing gene inserts at the *attB* site used by the mycobacteriophage L5 for insertion. The *attB* site is located within the 3' end of the tRNA gene, *glyV* but the gene itself is not disrupted, as the *attB* site is retained due to the formation of *attL* and *attR*. In this way, the re-introduced gene is inserted at a different position to that in the wild type strain (Figure 6.1).

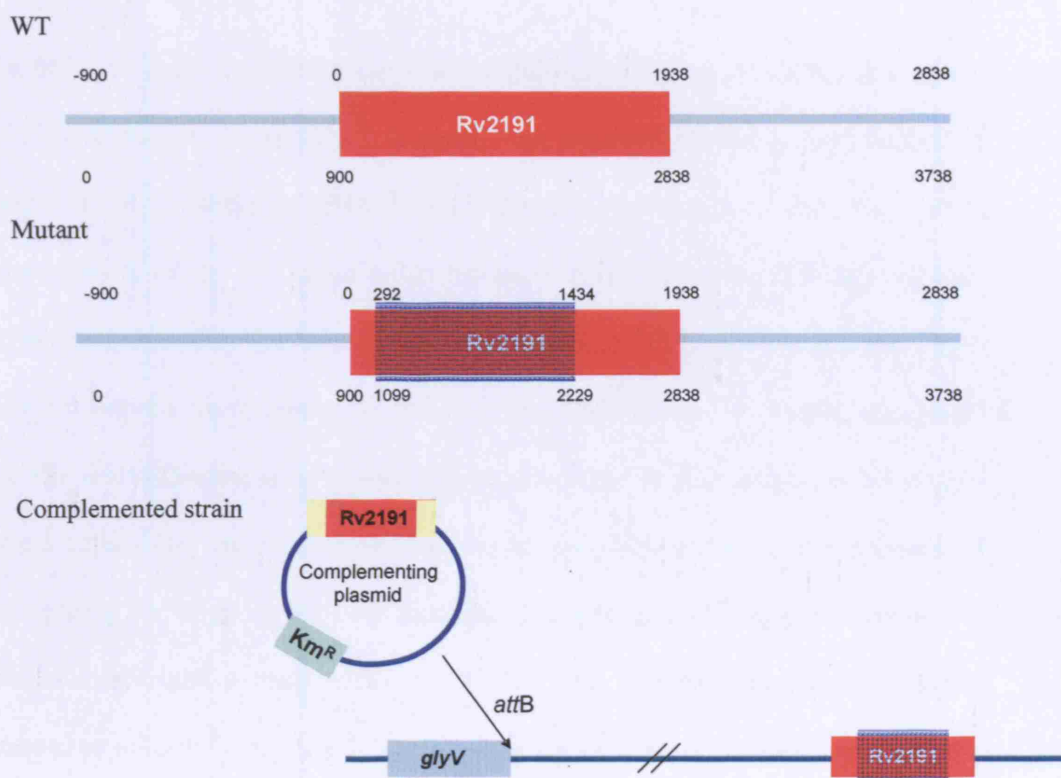


Figure 6.1: Comparative locations of deleted and complemented regions for Rv2191

The WT schematic shows the location of the gene for comparison with the mutant and complemented strains. The mutant schematic demonstrates the area of the gene which was deleted in order to construct the mutant strain. A section of the gene from 292-1434 bp was deleted (blue area), creating a strain lacking in 1143 bp. The deleted region encompasses the majority of the *Rv2191* gene (*Rv2191*= 1938bp).

The schematic for the complemented strain shows the complementing plasmid containing the gene itself (red) and its flanking region (yellow). The complemented gene fragment incorporates a flanking region of 640 bp on the 3' side and 173bp on the 5' side of the gene. Incorporation of the plasmid confers kanamycin resistant. As the entire mutated segment has been reinstated, the mutant strain is reverted to WT. The complement inserts at a new position in the genome, into the *attB* site of the *glyV* gene, used by the L5 phage for integration. The above diagram is not to scale.

6.3 Viability studies

In order to fully investigate the role of the mutated gene, Rv2191, in survival during exposure to oxidative damage, the viability of the mutant following exposure to a number of DNA damaging agents was examined and compared to the viability of the WT strain under the same conditions. This is a very rigorous assay and quantifies the level of survival, measured in cfu, following a prolonged exposure to the damaging agent. It was important to further investigate the results of the disc diffusion assay in this way as although the disc assay is a commonly used screen for assessing basic phenotype and was suited to the purpose of comparing a large group of mutants, it does not distinguish between a bacteriostatic and a bactericidal effect. The disc diffusion susceptibility assay measures inhibition of growth in the presence of a specific agent, whereas the viability assay measures survival following exposure to the agent.

Variations in susceptibility to different agents causing oxidative damage, and depending on the phase of growth have been reported in yeast (Mutoh *et al.*, 2005). Therefore, this emphasizes that although the disc diffusion assay is a good basic screen, the results should be interpreted with caution. Additionally, different results or phenotypes can be obtained from different ROI inducing chemicals, the reasons for this possibly being variation in the mechanism of action.

The focus of this investigation was on the effects of oxidative damage, and to test this, the mutant strain was exposed to hydrogen peroxide, tertiary butyl hydroperoxide (TBHP), menadione and diamide.

If survival of *Mtb in vivo* is dependent upon survival in activated macrophages, it is likely that the bacteria must withstand nitrosative damage as well as that incurred from ROIs. Activated macrophages produce RNIs, which cause a range of damage to bacterial DNA, lipids and proteins. Therefore, as well as investigating the effect of oxidative damage on viability, it was decided to expose the mutant strain to acidified sodium nitrite, a DNA damaging agent which produces reactive nitrogen intermediates. As mentioned previously, the *uvrB* mutant is sensitive to nitrosative damage (Darwin and Nathan 2005), implicating the NER pathway in this kind of repair. As Rv2191 is thought to be involved in NER, this suggests that the Rv2191 mutant strain may also show decreased viability following exposure to acidified sodium nitrite.

6.3.1 Oxidative stress

6.3.1.1 Hydrogen Peroxide

Hydrogen peroxide, an inorganic peroxide, is a powerful oxidizing agent, which acts by oxidising cellular components, making the intracellular environment favourable for the production of superoxide radicals.

As explained previously in chapter 1, in the presence of free cellular Fe^{2+} and other transition metal ions, hydrogen peroxide can give rise to highly reactive hydroxyl radicals via the Fenton reaction (Fenton 1894). These hydroxyl radicals react with DNA bases and sugars to cause damage to the DNA in the form of modifications and strand breaks. Alternatively, hydroxyl radicals can be formed

via the Haber Weiss reaction, whereby the superoxide radical (O_2^-), reacts with hydrogen peroxide directly.

6.3.1.2 Tertiary-butyl hydroperoxide

Tertiary-butyl hydroperoxide (TBHP) is an organic peroxide, commonly used as an *in vitro* model for oxidative stress. There are a number of lipid peroxides that are formed in cells as part of their defence mechanisms, and t-butyl hydroperoxide, although not itself a naturally occurring peroxide, is regarded as a good oxidant for modelling the effects of these lipid compounds.

TBHP has a similar basic structure to hydrogen peroxide, the difference being that one of the hydrogen atoms is replaced with an organic constituent, i.e. a carbon atom attached to 3 methyl groups. This has a significant bearing on the action of the peroxide and organic peroxides are very powerful oxidizing agents, releasing oxygen with a rapid decomposition, and can be used to initiate free radicals over a range of conditions.

6.3.1.3 Menadione

As discussed previously in chapter 4, menadione (a quinone), is a redox recycling agent which mimics the effects of oxidative stress and produces superoxide radicals by redox cycling within the cell.

6.3.1.4 Diamide

Diamide is a thiol specific oxidant, which creates a source of intracellular ROI. Diamide causes the toxic formation of cytoplasmic disulfides in low molecular weight thiols as well as in proteins (Kosower and Kosower 1995). In *E. coli*, exposure to diamide causes intracellular glutathione, an anti-oxidant, to become oxidised and thus inhibits its action. This renders the cell more susceptible to the effects of ROIs. Mycobacteria do not produce glutathione, and instead contain mycothiol (Newton *et al.*, 1996). It has been reported in *Streptomyces coelicolor*, that the *sigR* system is involved in the expression of the thioredoxin system in response to diamide and cytoplasmic bond formation (Paget *et al.*, 1998). This system is also functioning in Mtb (Paget *et al.*, 1998).

6.3.2 Nitrosative stress

Acidified sodium nitrite is commonly used as a source of nitric oxide *in vitro* to mimic the potential effects of NO *in vivo*. NO at a suitable concentration can have a potent bactericidal effect on Mtb and is one of the main defence mechanisms of activated macrophages. NO production is dependent on the expression of inducible nitric oxide synthase, iNOS, which is expressed in granulomas in the lungs of Mtb infected mice (Flynn *et al.*, 1998).

In the acidic environment of the phagosomes within activated macrophages, pH 5.4- 5.5, (Schaible *et al.*, 1998), nitric oxide is readily oxidized to nitrite, which is then partially protonated to nitrous acid. The nitrous acid then dismutates to form the radicals NO and $\cdot\text{NO}_2$ (Stuehr and Nathan 1989). $\cdot\text{NO}_2$ can be further oxidized to nitrate (NO_3^-), (Nathan and Shiloh 2000).

NER, specifically its component UvrB, has been implicated in the repair of damage caused by exposure to nitrosative stress (Darwin and Nathan 2005) and a UvrB mutant was found to be hyper-susceptible to NO *in vitro* (Darwin *et al.*, 2003). However, there are a number of mechanisms and alternative pathways in place to prevent the damage from exposure to RNI from occurring in the first instance, such as BER and mycothiols with antioxidant activity (Anderberg *et al.*, 1998).

Twelve genes have been identified which are implicated in repair of RNIs. These mutant strains were hypersensitive to the effects of acidified sodium nitrite, implicating the genes in question in survival against nitrosative stress (Darwin *et al.*, 2003). Although Rv2191 is not one of these genes, the fact that *uvrB* is one of these genes and has been shown to play a role in survival may further implicate the pathway and hints at a potential role for Rv2191 here as well.

6.4 Viability assays

The viability assay was performed according to a protocol adapted from one kindly provided by Dr Lucinda Rand (Novartis, Singapore). In short, the strain being tested was incubated with the selected compound for a designated amount of time in a 96 well plate and then serially diluted and plated out. After 15 days, the number of colony forming units was recorded as a measure of the viability of the strain (Figure 6.2). The mutant, wild type and complement were investigated in this way and the results compared. The concentrations and incubation times used were standardised based on assays conducted with the wild type strain prior to the experiment examining the other strains.

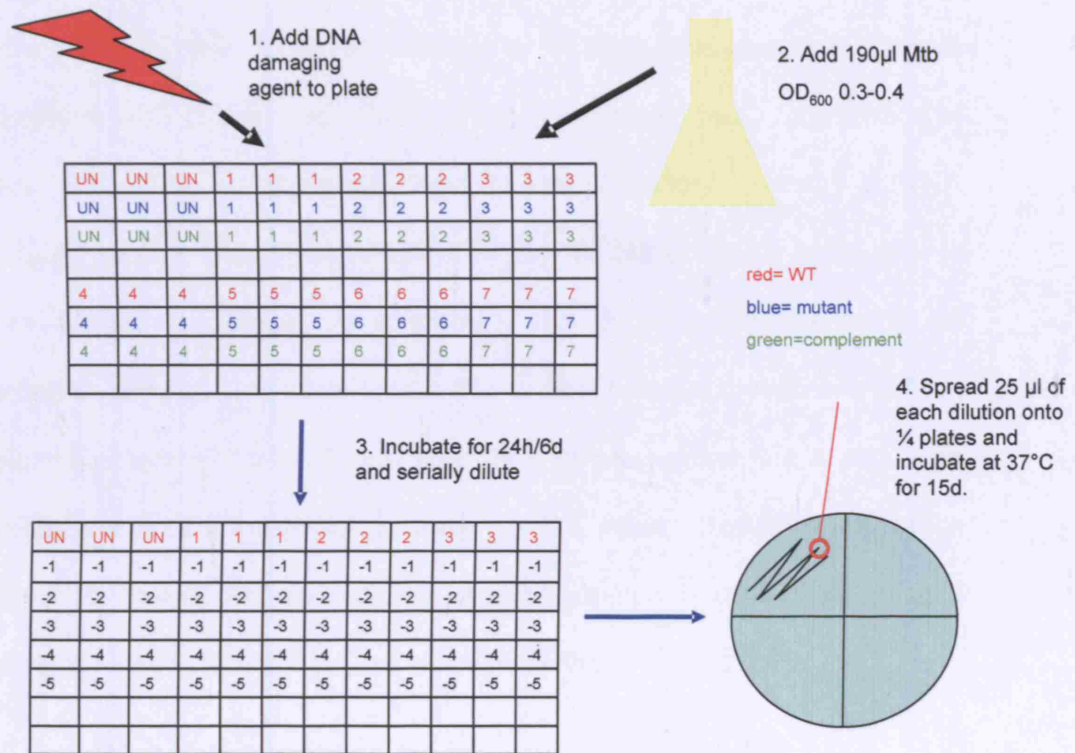


Figure 6.2 : Viability assay methodology

Strains were grown to an OD₆₀₀ of 0.3-0.4. 10 µl of DNA damaging compound or Dubos media for the untreated control were added to each well followed by 190 µl of Mtb. Plates were incubated at 37°C for the designated time (24h or 6d). At the determined time point, plates were removed from the incubator for serial dilution. Serial dilution was carried out using saline with a multi-channel pipette. 25 µl from 10⁻¹ → 10⁻⁴ dilutions were spread on quarter plates from treated wells containing mutant strains. For the wild type and complemented strains and all untreated controls, 10⁻² → 10⁻⁵ dilutions were plated out. Plates were allowed to dry and incubated at 37°C for 15d.

Each experiment was carried out in triplicate (3 biological replicates) with 3 technical replicates.

— roller bottle

6.4.1 Standardisation of Viability experiment

In the protocol obtained and in the literature, the microplate assay is performed with a 6 day incubation following addition of the DNA damaging agent (Darwin and Nathan 2005; Rand 2006). This is a longer incubation than in the method of investigating viability previously used in our laboratory, whereby a DNA damaging agent is added to a 40-100ml culture for 24h in a roller bottle prior to serial dilution and plating. It is probable that the longer incubation in the microplate protocol is to allow for the fact that the bacterial growth in a 96 well plate will be slower as the culture is static. However, to confirm this, as part of the standardisation of the experiment using the WT strain, 2 different incubation times were trialed, 24h and 6d. The standardisation was carried out using 2 biological replicates, each with 2 technical replicates.

The damaging compounds chosen were selected to reflect a broad range of oxidative stress conditions. Acidified sodium nitrite was used to reflect conditions induced by nitrosative stress. Here, the untreated control was re-suspended in acidified Dubos media (pH5.4) to control for the effect of pH alone. A range of concentrations of the DNA damaging compounds were tested based on the concentrations used in previous studies, both within the laboratory and reported in the literature (table 6.1):

The optimal effect desired is a difference in viability of around 1 log, to decrease the viability sufficiently to be sure that the compound is effective, whilst allowing for the detection of any heightened sensitivity in the mutant. A difference of much

more than 1 log in the WT strain may produce complete killing in a hyper-susceptible mutant strain.

Table 6.1: Compounds and concentrations tested in the standardisation of the viability assays

Concentrations in bold indicate those used in subsequent viability assays.

Compound	Concentration 1	Concentration 2	Concentration 3
Menadione	100 μM	250 μ M	500 μ M
H ₂ O ₂	2mM	5mM	10mM
TBHP	100 μ M	250 μM	500 μ M
Diamide	5mM	10mM	20mM
Acidified sodium nitrite	1mM	3mM	6mM

At 24 h, although there was a decrease in survival of approximately 1 log compared with the untreated control after exposure to 100 μ M menadione, there was no further decrease at higher concentrations (Figure 6.3). This lack of a dose response suggested that a clearer result might be obtained for this agent following exposure for a longer time.

In contrast, the difference between the untreated sample and those treated with varying amounts of H₂O₂ for 24 h was clear with a good dose response observed (Figure 6.4). At 2mM, there was a 0.5-1 log decrease in viability compared with the untreated sample. At 5mM, survival decreased by 2 log, and at 10mM, survival decreased by 2.5 log. The decrease at a concentration of 2mM H₂O₂ looks promising for further viability experiments.

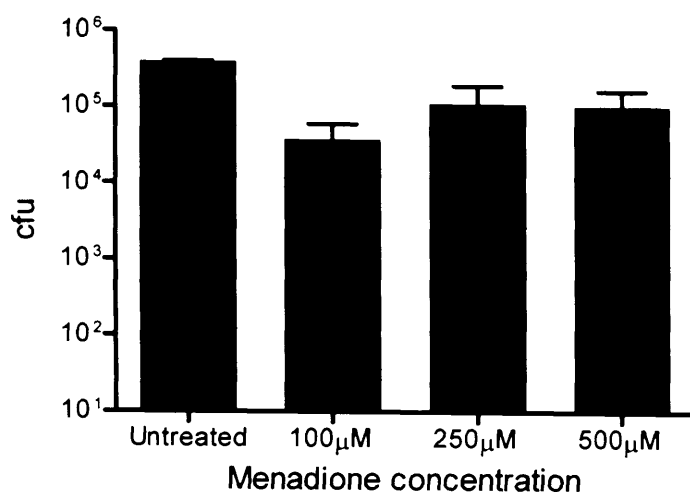


Figure 6.3: Viability standardisation: 24h exposure to menadione

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of menadione (x axis) for 24h and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. At 24h, there is a slight decrease in viability following exposure to all 3 concentrations of menadione compared to the untreated sample but this is not reflected by a clear dose response.

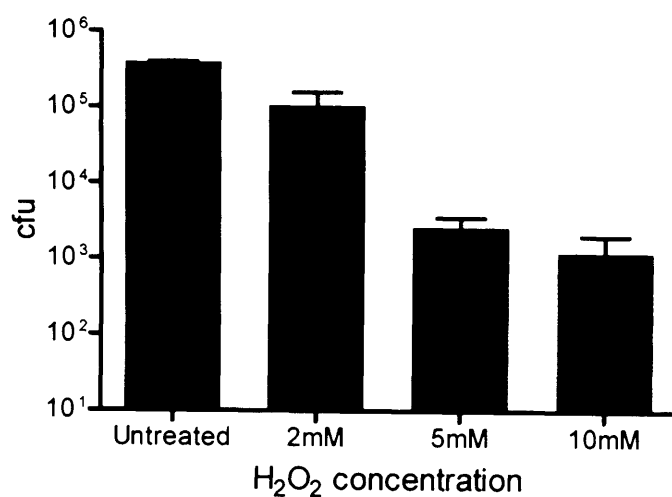


Figure 6.4: Viability standardisation: 24h exposure to H₂O₂

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of H₂O₂ (x axis) for 24h and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. A clear dose response is apparent in the viability of the WT strain following exposure to varying concentrations of H₂O₂ for 24h.

At 24 h, exposure to TBHP resulted in a marked decrease in viability at all of the concentrations tested (Figure 6.5). There did appear to be a slight dose response. Survival following exposure to 100 and 250 μ M TBHP was similar and decreased by 2.5 log, and following exposure to 500 μ M, decreased by 3 log.

Survival following the 24 h exposure to diamide was also affected but only at the higher concentrations tested (10 and 20mM) with a clear dose dependent response (Figure 6.6). At 10mM, survival decreased by 0.5 log, and decreased by 1.5 log at 20mM.

There was no significant difference seen between culture exposed to acidified sodium nitrite for 24 h and the low pH control (Figure 6.7). As these concentrations are commonly reported for this assay in the literature, it is likely that the exposure time needs to be increased to see a clearer dose response.

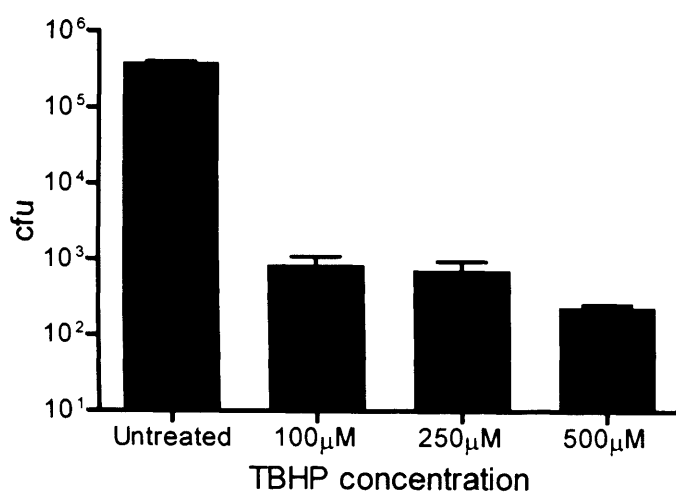


Figure 6.5: Viability standardisation: 24h exposure to TBHP

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of TBHP (x axis) for 24h and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. The graph shows a clear decrease in viability of the WT strain following 24h exposure to all the tested concentrations of TBHP.

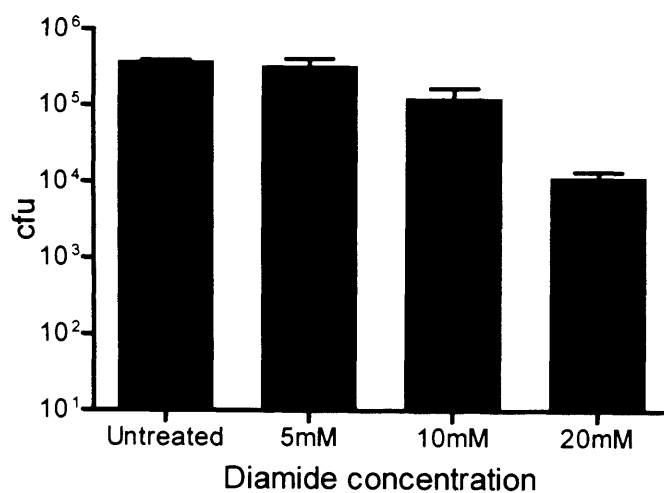


Figure 6.6: Viability standardisation: 24h exposure to diamide

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of diamide (x axis) for 24h and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. There is a decrease in viability of the WT strain following exposure to 10 and 20 mM diamide. There appears to be no difference between viability following exposure to 5mM diamide, compared with the untreated sample.

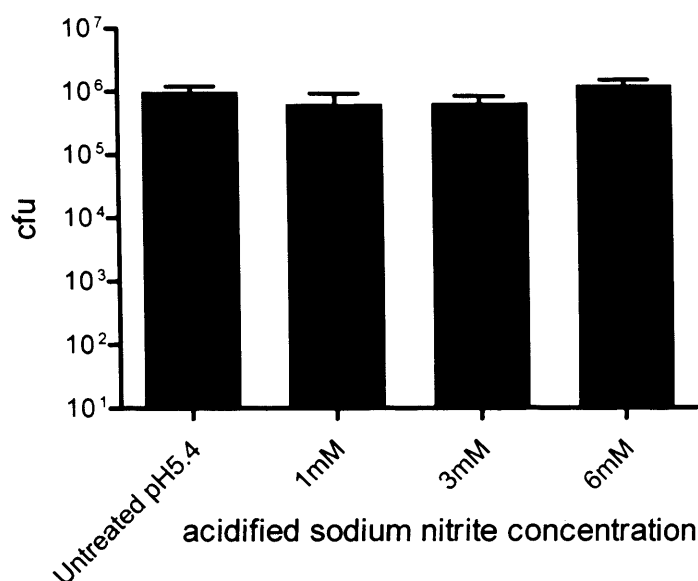


Figure 6.7: Viability standardisation: 24h exposure to acidified sodium nitrite

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of acidified sodium nitrite (x axis) for 24h and comparing the number of cfu (y axis) to the untreated control, pH5.4, following serial dilution and plating.

There are no differences in viability between untreated and treated WT samples following exposure to varying concentrations of acidified sodium nitrite. As the acidified sodium nitrite is at pH5.4, the untreated control culture is also at pH5.4. This is to ensure that any change in viability is due to the effects of the damaging compound and not due to the acidic pH.

Following the 6 d exposure to menadione (Figure 6.8), there was a good dose response observed, with 100 μM causing an approximately 1 log decrease in survival. This is the optimal decrease in terms of standardisation and this concentration was therefore chosen to test viability of the mutant strain. Exposure to 250 μM menadione caused a 2 log decrease in survival, and exposure to 500 μM caused a 6 log decrease.

The exposure to H_2O_2 for 6 days (Figure 6.9) caused an approximately 1 log decrease in survival at 2mM, and was therefore chosen as the concentration to test mutant viability with. A 1.5 log decrease in survival was observed following exposure to 5mM H_2O_2 , and a 4.5 log decrease at 10mM.

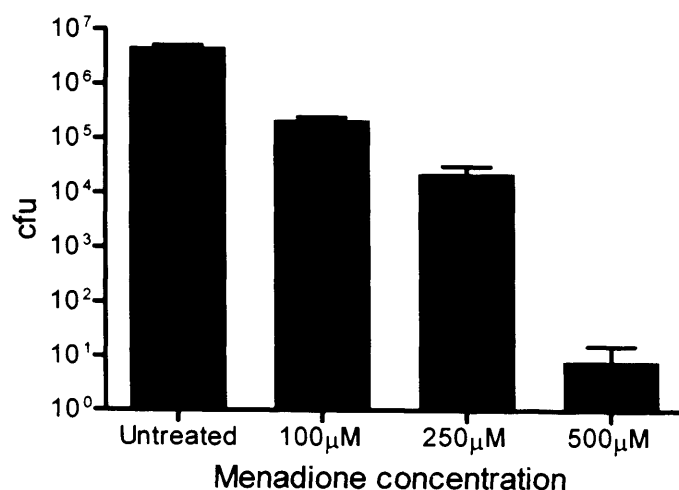


Figure 6.8: Viability standardisation: 6d exposure to menadione

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of menadione (x axis) for 6d and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. There is a clear dose related difference in viability of the WT strain following exposure to different concentrations of menadione, compared to the untreated sample.

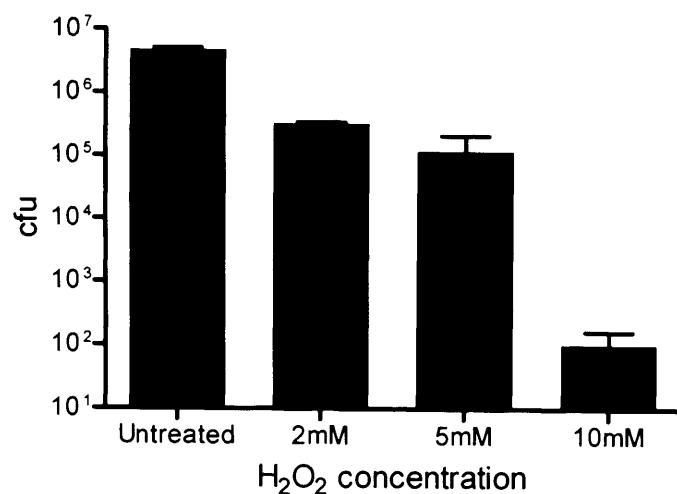


Figure 6.9: Viability standardisation: 6d exposure to H₂O₂

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of H₂O₂ (x axis) for 6d and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. There is a clear dose related difference in viability of the WT strain following exposure to different concentrations of H₂O₂, compared to the untreated sample.

Although survival following exposure to TBHP was reduced following a 6d exposure (Figure 6.10), there did not appear to be a dose related response, rather all the concentrations showed the same effect. It is possible that after a longer incubation time, there is a threshold concentration above which any amount will have a similar effect, regardless of the concentration up to a certain point. Perhaps surprisingly, the decrease in viability observed at 6d was less marked than that seen following exposure for 24h. This could potentially reflect an issue with stability of the compound. 250 μ M was selected as the concentration used to investigate viability in subsequent experiments.

Exposure to the varying concentrations of diamide caused a dose related decrease in survival (Figure 6.11). At 5mM and 10mM, survival fell by 1 log. However, at 20mM, survival decreased by almost 4 log. 10mM was selected as the concentration used to investigate viability in subsequent experiments, as it caused a 1 log decrease in survival.

Exposure to acidified sodium nitrite caused a relatively uniform decrease in survival following exposure for 6 days (Figure 6.12). Survival fell by approximately 1 log at 1mM and 3mM sodium nitrite. At 6mM, bacterial survival decreased by almost 2 log. 3mM was chosen as the concentration used to investigate viability in subsequent experiments.

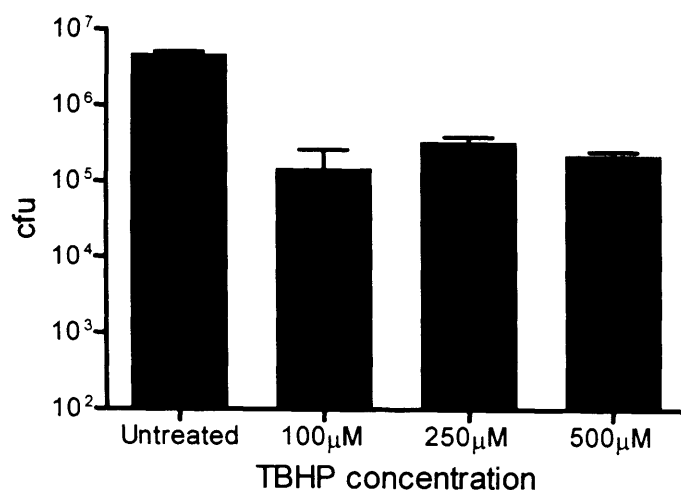


Figure 6.10: Viability standardisation: 6d exposure to TBHP

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of TBHP (x axis) for 6d and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. Despite the fact that there is a clear decrease in viability of the WT strain following exposure to all the tested concentrations of TBHP, this does not appear to be dose related and remains relatively static between 100 and 500 μ M.

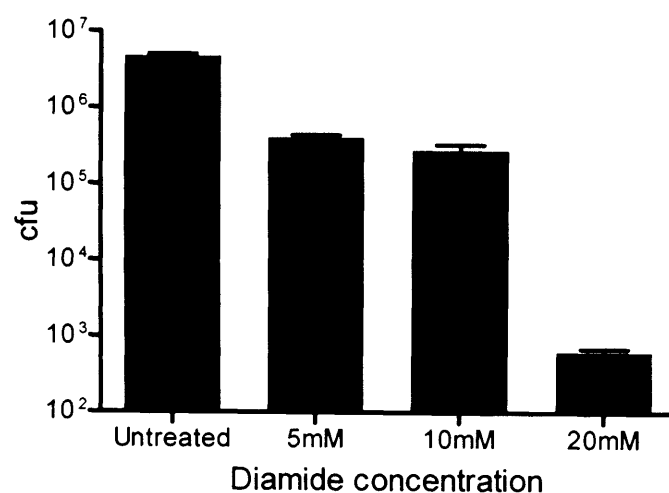


Figure 6.11: Viability standardisation: 6d exposure to diamide

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of diamide (x axis) for 6d and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. There is a clear dose related decrease in viability of the WT strain following exposure to the varying concentrations of diamide. There is a marked decrease in viability between 10 and 20 mM.

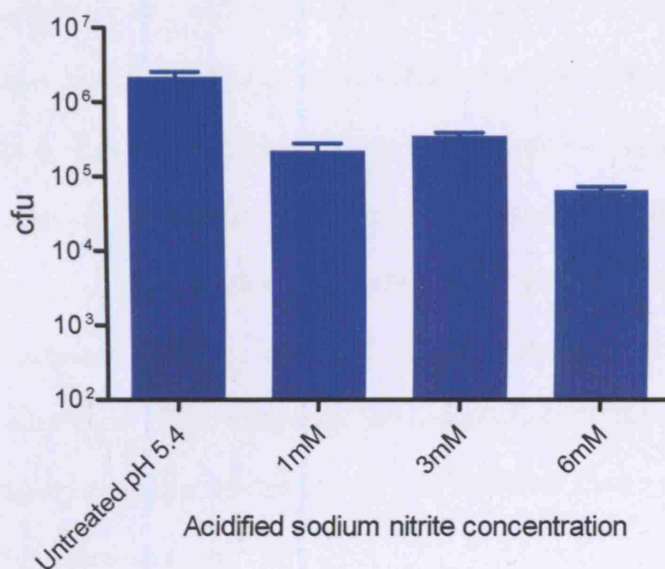


Figure 6.12: Viability standardisation 6d exposure to acidified sodium nitrite

Viability of the WT strain was measured by exposing the culture to a number of different concentrations of acidified sodium nitrite (x axis) for 6d and comparing the number of cfu (y axis) to the untreated control, following serial dilution and plating. There is a decrease in viability of the WT strain following exposure to the varying concentrations of sodium nitrite. At 1 and 3 mM, the difference is similar, whereas it drops further at 6mM.

Based on the results obtained from the standardisation experiments, it was decided to compare the viabilities of the different strains following exposure to damaging agents for 6d, with an additional test for viability following exposure to TBHP after 24h. An additional test to measure viability following exposure to H₂O₂ after 24 h was also performed in case of potential instability of H₂O₂. Stability and potential degradation of the damaging compound must also be taken into account, although this did not appear to be a major issue here as all the compounds caused decreased viability compared to the untreated control under at least some conditions. If the compound had degraded or displayed limited stability, it would have been expected that the viability of treated samples would be similar to that of untreated controls.

6.4.2 Viability Assay: Rv2191

The wild type, mutant and complemented strains were subjected to each of the DNA damaging agents at the concentrations determined in the standardisation assay, with a 6d exposure for all. In the case of TBHP and H₂O₂, a 24h incubation was also performed. Experiments were carried out in triplicate, with 3 repeats, thus generating 9 sets of data per damaging compound. Data were normalised by expressing treated values, determined as cfu, as a percentage of the untreated value for that particular strain, performed in parallel. This made comparison between compounds clearer.

6.4.2.1 Menadione

There was no statistically significant difference in viability between the wild type and mutant strains following a 6d incubation with menadione (Figure 6.13). This is somewhat surprising, given that menadione inhibited growth of the Rv2191 mutant in the disc diffusion assay. However, as mentioned previously, the assays differ fundamentally. The disc diffusion assay is used to investigate growth in the presence of a particular compound, and therefore the strain must repair this damage to allow further growth. It is used to investigate the bacteriostatic effect of the compound in question. The viability assay is used to investigate survival following exposure to a chemical. This is the bacteriocidal effect. Therefore the viability assay looks at viable numbers of bacteria, as determined by growth following exposure to menadione, as opposed to the effect of menadione on growth in its presence.

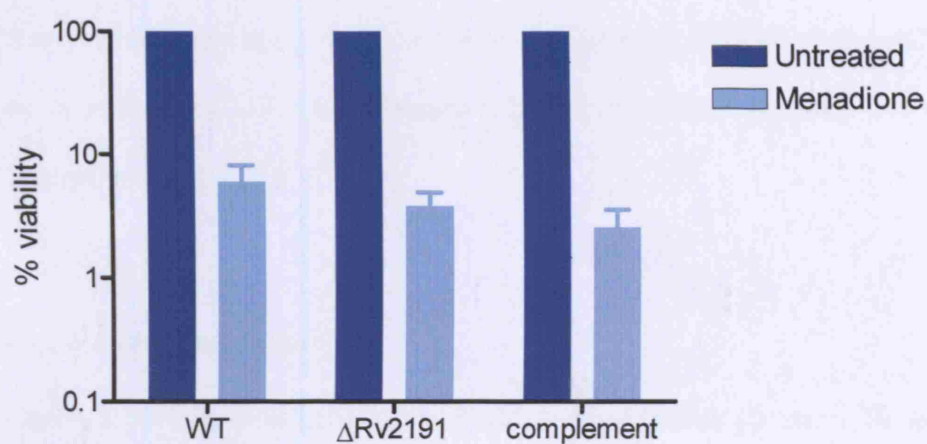


Figure 6.13: Viability of Rv2191 mutant strain following 6d exposure to menadione

The WT, mutant and complemented strain were exposed to menadione for 6d, serially diluted and plated out. cfu were counted and viability calculated by expressing the cfu from the treated sample, as a percentage of the cfu from the untreated sample for each strain.

There appears to be a slight difference between the viability of the mutant strain compared with that of the WT strain following 6d exposure to 100 μ M menadione. This is not however statistically significant and not reflected by a decrease in viability compared to the complemented strain. Although there appears to be a difference between the WT and complemented strains, this is not statistically significant.

6.4.2.2 H₂O₂

There was no statistically significant difference between viability of the wild type and mutant strains following exposure to hydrogen peroxide for either 24h or 6d (Figures 6.14 and 6.15).

6.4.2.3 T-butyl hydroperoxide

There was a decrease in viability in the wild type strain seen after both 24h and 6d incubation with TBHP (Figures 6.16, 6.17). However, only the difference at 6d was deemed statistically significant (Unpaired T test, $p=0.0103$). In the complemented strain, this result is clearly reverted back to the WT level. Although the difference between the mutant and WT strains was not statistically significant at 24h, the difference between the mutant and complement is significant (Unpaired T Test, $p=0.0006$), which suggests that there is a true difference here.

This result is particularly exciting as it supports a potential role for Rv2191 in survival following exposure to oxidative damage.

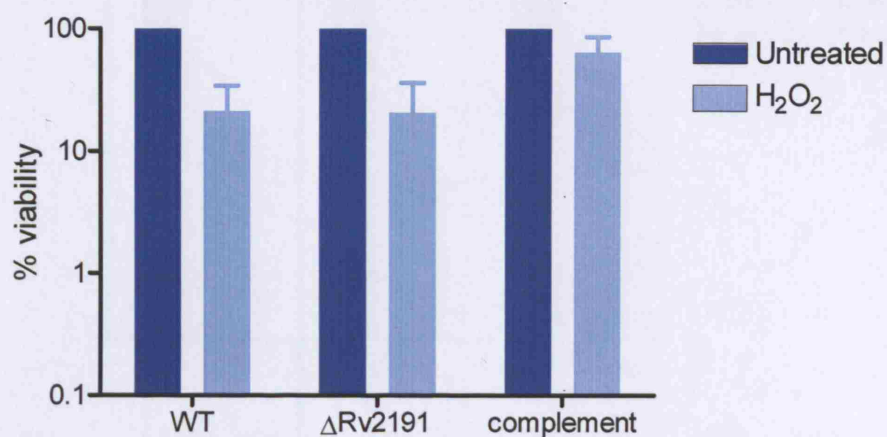


Figure 6.14: Viability of Rv2191 mutant strain following 24h exposure to H₂O₂

The WT, mutant and complemented strain were exposed to H₂O₂ for 24h, serially diluted and plated out. cfu were counted and viability calculated by expressing the cfu from the treated sample, as a percentage of the cfu from the untreated sample for each strain. There is no difference between viability of the mutant and WT strains following a 24h exposure to 2mM H₂O₂.

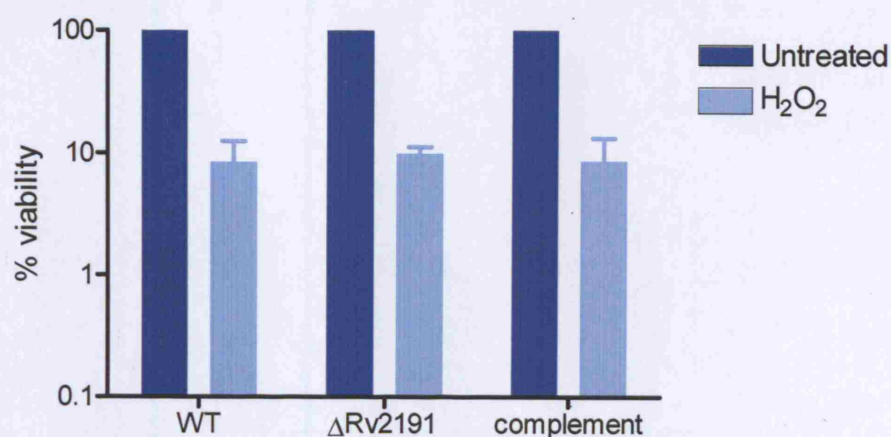


Figure 6.15: Viability of Rv2191 mutant strain following 6d exposure to H₂O₂

The WT, mutant and complemented strain were exposed to H₂O₂ for 6d, serially diluted and plated out. cfu were counted and viability calculated by expressing the cfu from the treated sample, as a percentage of the cfu from the untreated sample for each strain. There is no difference between viability of the mutant and WT strains following a 6d exposure to 2mM H₂O₂.

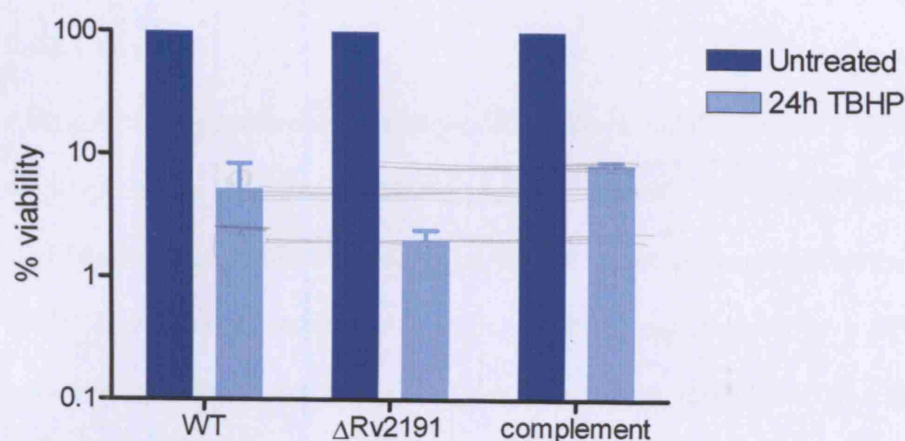


Figure 6.16: Viability of Rv2191 mutant strain following 24h exposure to TBHP

The WT, mutant and complemented strain were exposed to TBHP for 24h, serially diluted and plated out. cfu were counted and viability calculated by expressing the cfu from the treated sample, as a percentage of the cfu from the untreated sample for each strain. Although a clear difference is apparent between WT and mutant strains following 24h exposure to 250 μ M TBHP, this is not statistically significant. However, there is a statistically significant difference between viability of the mutant and complemented strains (Unpaired T test, $p=0.0006$).

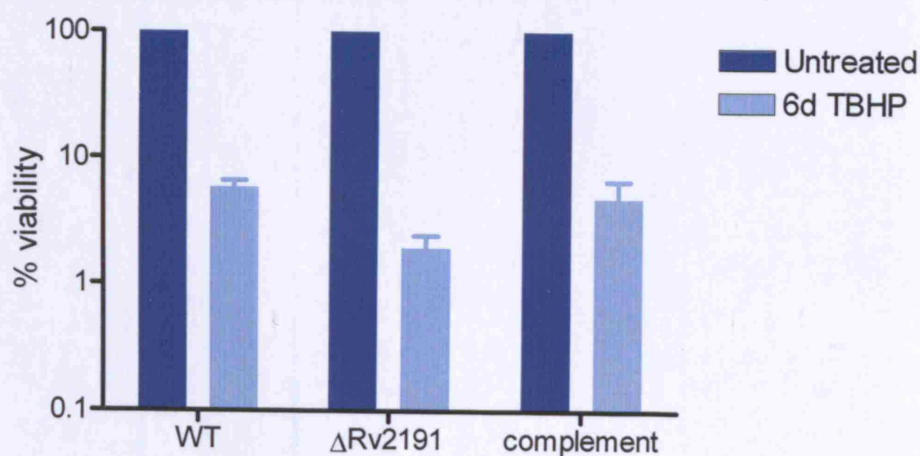


Figure 6.17: Viability of Rv2191 mutant strain following 6d exposure to TBHP

The WT, mutant and complemented strain were exposed to TBHP for 6d, serially diluted and plated out. cfu were counted and viability calculated by expressing the cfu from the treated sample, as a percentage of the cfu from the untreated sample for each strain. The difference in viability between WT and mutant strains following exposure to 250 μ M TBHP is statistically significant (Unpaired T test $p=0.0103$). The phenotype is reverted to WT in the complemented strain.

6.4.2.4 Diamide

Although there appeared to be a slight difference in viability between mutant and wild type strains following a 6d incubation with diamide, this was not statistically significant (Figure 6.18). The decreased viability in the mutant strain is reverted to the WT level in the complemented strain. As there does appear to be a difference between WT and mutant strains, this could be further investigated at a range of concentrations and or incubation times. The potential difference seen here may hint at a slight decrease in viability following exposure to ROI.

6.4.2.5 Acidified Sodium nitrite

There was no difference in viability between mutant and WT strains after exposure to acidified sodium nitrite for 6d (Figure 6.19).

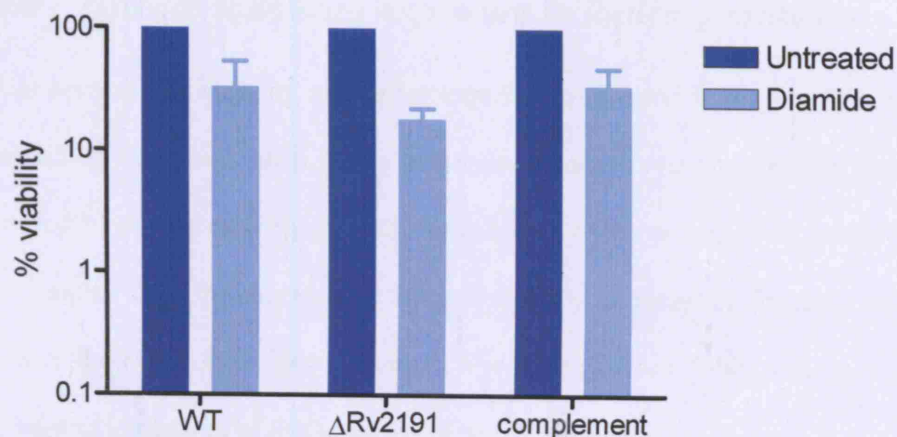


Figure 6.18: Viability of Rv2191 mutant strain following 6d exposure to diamide

The WT, mutant and complemented strain were exposed to diamide for 6d, serially diluted and plated out. cfu were counted and viability calculated by expressing the cfu from the treated sample, as a percentage of the cfu from the untreated sample for each strain. Although there appears to be a slight difference between WT and mutant strains following a 6d exposure to diamide, this is not statistically significant. The observed decrease in mutant viability is reverted to WT in the complemented strain.

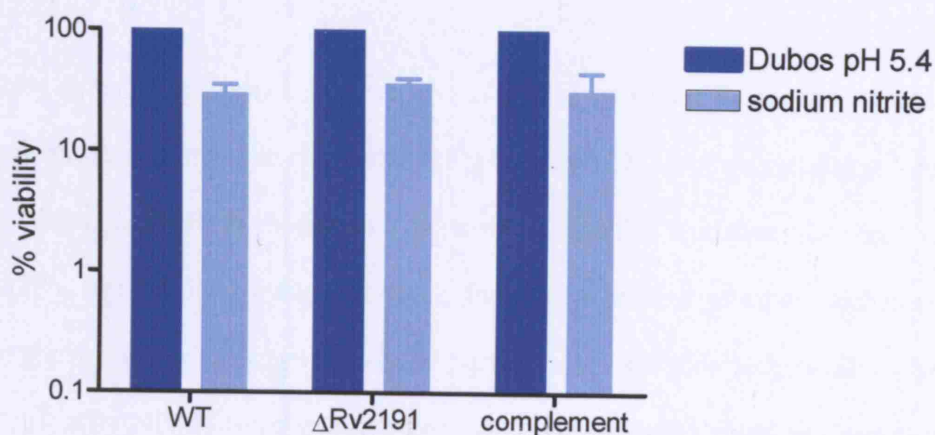


Figure 6.19: Viability of Rv2191 mutant strain following 6d exposure to acidified sodium nitrite

The WT, mutant and complemented strain were exposed to acidified sodium nitrite for 6d, serially diluted and plated out. cfu were counted and viability calculated by expressing the cfu from the treated sample, as a percentage of the cfu from the untreated sample for each strain. There is no difference between viability of the WT and mutant strains following exposure to 3 mM sodium nitrite pH 5.4.

6.5 Growth following exposure to ionizing radiation

The decrease in viability seen after exposure to T-butyl hydroperoxide, and the inhibition of growth seen in the presence of menadione suggested a phenotype caused by agents delivering oxidative damage, which causes DNA strand breaks. In light of this, the mutant's viability following exposure to ionizing radiation, which induces double strand breaks (Ward 1988; Ward 1990) was investigated. (For standardisation of this experiment see chapter 5).

WT and mutant strains were grown to exponential phase (OD_{600} 0.3-0.4) and exposed to gamma radiation from a Caesium source (^{137}Cs). The cultures were returned to rolling at 37°C overnight, serially diluted and viability assessed by counting the number of colony forming units after incubation for 15 days at 37°C, and comparing the values for wild type and mutant.

There was no difference in viability following exposure to gamma radiation between mutant and wild type strains (Figure 6.20). This is not a surprising result, as although NER is implicated in survival against oxidative damage, and additionally UV light induced damage, there are a number of other mechanisms for repairing double strand breaks, in particular homologous recombination and additionally NHEJ. Even in the event that these pathways could not repair the damage caused by exposure to ionizing radiation, and NER did play a significant role, it is probable that UvrC would be able to repair the damage, with Rv2191 playing a minimal role.

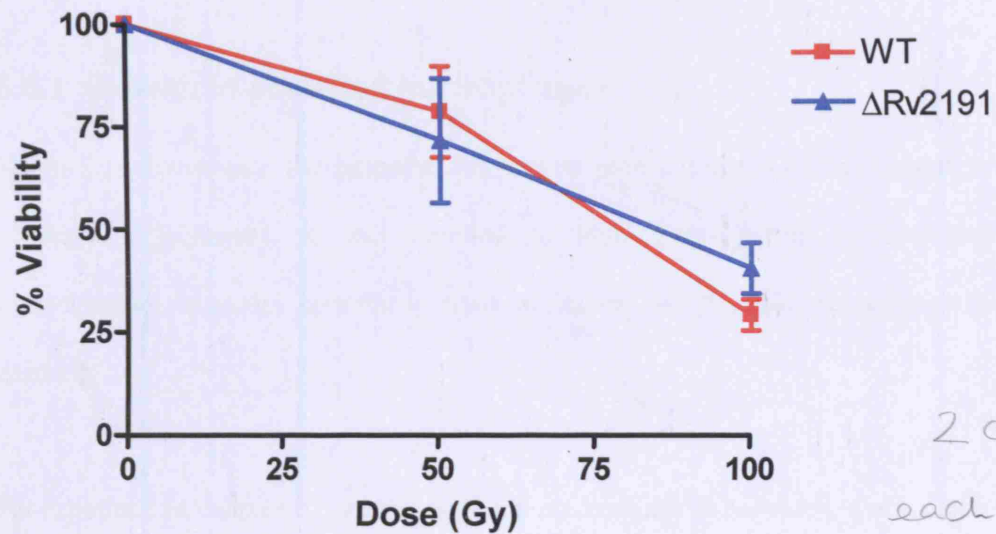


Figure 6.20: Viability of Rv2191 mutant strain following exposure to ionizing radiation

There is no significant difference in viability following exposure to ionizing radiation between WT and mutant strains. Cultures were exposed to 50 and 100 Gy or left untreated and were incubated for 24h with rolling at 37°C following exposure to allow repair to take place. This was followed by serial dilution and plating.

Data were normalised by expressing cfu values for strains exposed to radiation as a percentage of the untreated values for the same strain.

6.6 *Growth in vivo*

6.6.1 Growth in activated macrophages

To further investigate the potential phenotype seen for the Rv2191 mutant in previous experiments, it was decided to investigate growth in activated macrophages, a model commonly used to screen for potential phenotypes in animals.

As explained in chapter 5, *in vivo*, once the macrophage is activated, the bacteria are exposed to a number of defence mechanisms which include the production of reactive oxygen intermediates (ROI) via phox and reactive nitrogen intermediates (RNI), via iNOS, which damage the bacterial DNA (Flynn and Chan 2001). The generation of superoxide and nitric oxide is approximately equal, which aids the production of the supremely destructive peroxynitrite (OONO[•]) (Nathan and Shiloh 2000). As described previously, Mtb are known to withstand these defence mechanisms.

Bone marrow derived macrophages were extracted from the femurs of female Balb/c mice and allowed to differentiate prior to inoculation with bacteria. The macrophages were incubated for 24h with IFN- γ and LPS and activation status was checked using a Griess assay (R&D systems), as previously explained in chapter 5. The Griess assay was repeated after washing the macrophages and replacing the media and additionally replacing IFN- γ and LPS. The results were consistent with those obtained previously.

There was no difference in growth between WT and Rv2191 mutant strains in activated macrophages (Figure 6.21). A *uvrB* mutant displayed a very slight deficit in growth in bone marrow derived activated macrophages (Darwin and Nathan 2005) and as UvrB is the main component of the NER pathway, it would be surprising if Rv2191 showed a marked attenuation of growth in activated macrophages. However, it is possible that a potential phenotype may be more noticeable in a more sophisticated animal infection model. For this reason, it was decided to investigate growth of the mutant strain in mice.

6.6.2 Growth in mice

Balb/c mice were infected with the WT, mutant and complemented strains. Mice were grouped (n=5) and at each time point (0, 30, 70, 100 days) mice from the relevant group were sacrificed by cervical dislocation. Lungs and spleen were homogenized and plated out, and the number of cfu recorded after a 15d incubation.

There were no differences in growth between wild type and mutant strains in mouse lungs (Figure 6.22) and mouse spleens (Figure 6.23), indicating that Rv2191 is not essential for infection in a mouse model.

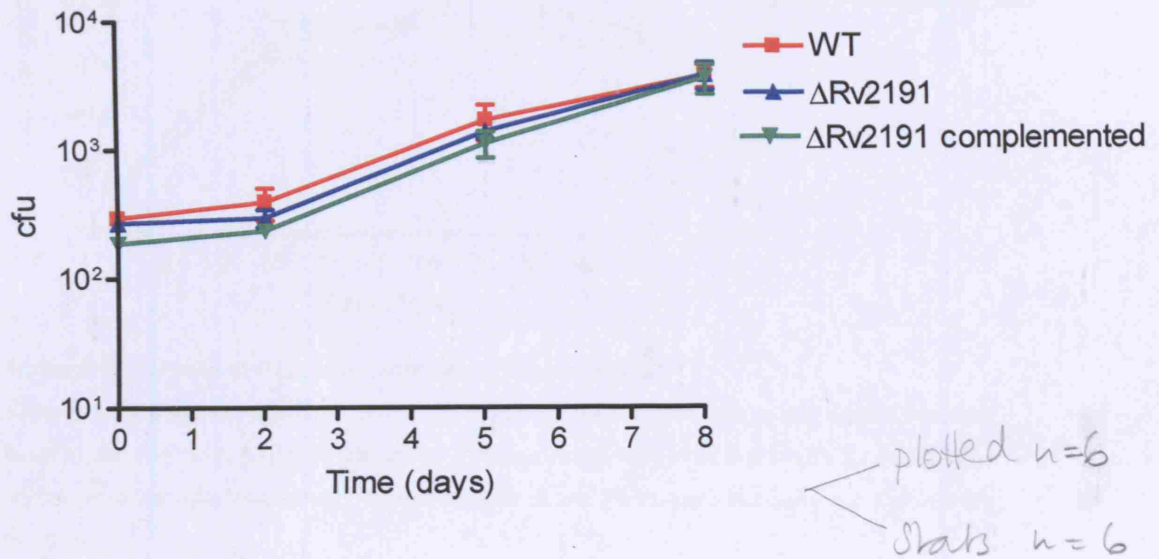


Figure 6.21: Growth of Rv2191 mutant strain in activated macrophages

Macrophages were activated by the addition of IFN- γ (100u/ml) and LPS (1 μ g/ml) 24h prior to inoculation with Mtb. The experiment was performed with 3 biological and 2 technical replicates. There is no difference in growth between the WT and mutant strains.

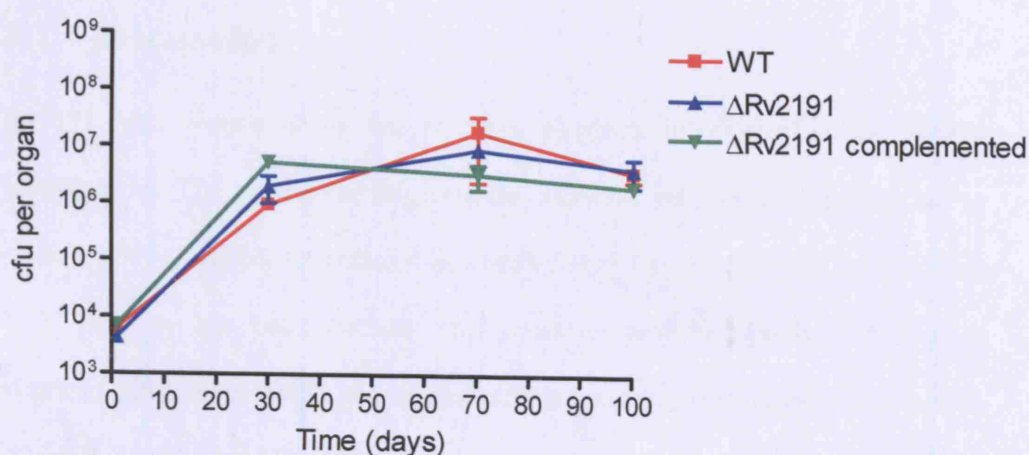


Figure 6.22: Growth of Rv2191 mutant strain in mouse lungs

There is no statistically significant difference in growth between WT and mutant strains in mouse lungs at any time point. At each time point, 5 mice infected with each strain were sacrificed. The organs were homogenized, serially diluted and plated out. Plates were incubated for 15d and cfu counted.

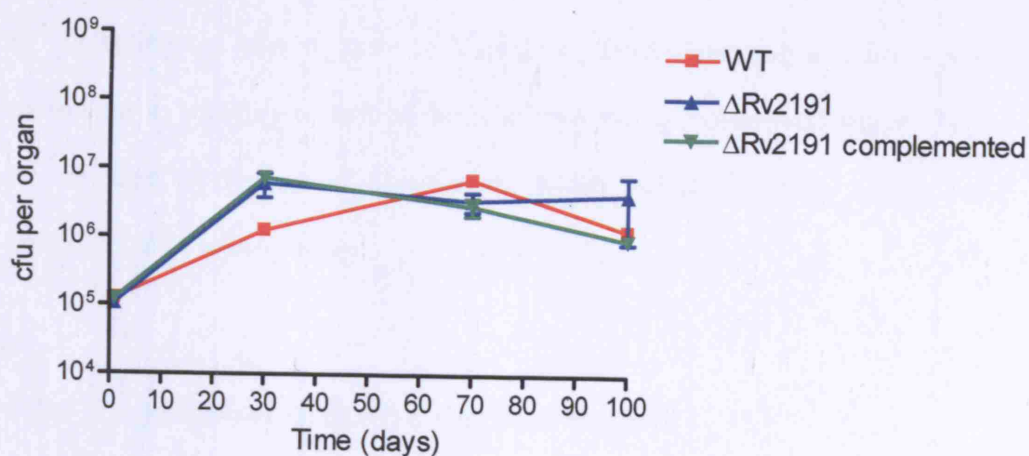


Figure 6.23: Growth of Rv2191 mutant strain in mouse spleens

There is no statistically significant difference in growth between WT and mutant strains in mouse spleens at d100. The WT strain appears to have a slight deficit in growth at d30 but this is corrected by d70. At each time point, 5 mice infected with each strain were sacrificed. The organs were homogenized, serially diluted and plated out. Plates were incubated for 15d and cfu counted.

6.7 Discussion

Rv2191 was chosen from the mutants screened in chapter 4 for further investigation. The reason for this was the apparent inhibition of growth in the presence of menadione and ofloxacin, coupled with its potential role in NER. The NER pathway has been recently implicated in survival against ROI *in vivo* (Darwin and Nathan 2005), and against nitrosative stress both *in vitro*, and *in vivo* (Darwin *et al.*, 2003; Darwin and Nathan 2005). An important role for NER in survival potentially implicates Rv2191 by default.

Therefore, the aim of this chapter was to thoroughly characterise the Rv2191 mutant, and consequently gain an understanding of the functions of Rv2191 itself, by subjecting the mutant strain to a range of DNA damaging conditions and comparing its viability to that of the wild type and complemented strains. This was pursued by a range of experiments, which included viability assays and investigation of growth *in vivo*.

6.7.1 Exposure to oxidative damage *in vitro*

The role of Rv2191 in viability following exposure to oxidative stress was confirmed by a statistically significant decrease in mutant viability following exposure to T-butyl hydroperoxide after a 6d incubation. Although a clear difference in viability was observed following exposure to diamide, this was not statistically significant.

Despite the fact that the mutant displayed heightened sensitivity to menadione in the disc diffusion susceptibility assay (Chapter 4), there was no similar decrease in viability observed following incubation with menadione. An important difference between the disc diffusion assay and the viability assay, is that the bacteria are grown on different media, which will have a bearing on their growth and survival. The disc diffusion assay is performed on solid media, whereas bacteria in the viability assay are exposed to the damaging agent in liquid culture and then plated out onto solid media following incubation. This has important implications on the potential diffusion of the drug, which may be inhibited by the solid agar or affected by components present in the media and is difficult to quantify.

It has been reported that another oxidative stress inducing agent, H_2O_2 , stopped the growth of *Saccharomyces pombe* yeast cells but did not affect their viability (Mutoh *et al.*, 2005), thus supporting the observation that the different physiological states of the cells in different growth media can have an effect on their sensitivity and/ or survival and give differing responses to the same agent presented or measured in a different way. It is also possible that the mutagenicity of the menadione increases with time, perhaps due to a build up of products, or as time goes on, the bacteria become less able to repair the ever increasing amount of damage. In the disc diffusion assay, the bacteria are incubated for 12 days, whilst in the viability assay, the incubation is for 6 days prior to serial dilution and plating. This difference in incubation time, may be a reason for the differing results from the assays. It is possible that although the mutagenic nature of

menadione is significant, this level of mutation arrests growth but does not affect survival. An increased dose may have a more significant effect on viability.

As described previously, a similar phenomenon was described by Darwin and Nathan (2005), where they reported that the *uvrB* mutant, despite not being sensitive to oxidative stress in a disc diffusion susceptibility assay *in vitro*, was sensitive to products of phox *in vivo*. This raises the issue that the gene may be involved in protection against a mixture of products and not just single ones, and potentially that the mutant may be susceptible to other ROI, which were not tested *in vitro* (Darwin and Nathan 2005).

Therefore, it is particularly interesting that the Rv2191 mutant had heightened sensitivity to killing by TBHP, even though statistically increased sensitivity to the other oxidative damage inducing agents was not observed. This may be explained by the differing mechanisms of actions used by the compounds to exert oxidative damage and has been reported in other studies (Paget *et al.*, 1998). It has been shown in yeast, using fluorescent stain, that the different reagents generate reactive oxygen species at different locations within the cell (Mutoh *et al.*, 2005). Also, the induction kinetics of genes involved in survival against these agents differs depending on the compound used, implying that the mechanisms used to survive them differ in some way (Mutoh *et al.*, 2005). It would be interesting to measure the ROI produced in each assay by the different chemicals; this could be done by using assays which quantify the presence of various oxidative products of ROIs. Additionally, further research could involve the use of Q-RT PCR to examine the extent of the damage to the DNA.

The role of ROI in host defence against Mtb and its contribution to bacterial DNA damage during infection remains controversial. The damaging effects of the reduction products of oxygen are undisputed, as is the ability of the activated macrophage to generate these products. However, some studies have questioned the importance of phox-derived ROI in TB infection (Cooper *et al.*, 2000) and in humans, it is questioned whether patients with Chronic Granulomatous Disease, ie lacking in the ability to generate ROI, are particularly susceptible to Mtb infection (Nathan and Shiloh 2000). Nonetheless, combining superoxide and nitric oxide leads to the production of peroxynitrite. Mice deficient in phox are unable to trigger production of ROI and are more susceptible to a number of inoculated pathogens (Jackson *et al.*, 1995; Pollock *et al.*, 2005), perhaps as they are unable to generate peroxynitrite. Although phagocyte oxidase, and therefore oxidative stress was not previously thought to be an essential defence mechanism for the host against Mtb (Cooper *et al.*, 2000), it is possible that the effects of phox are normally averted in some way by *uvrB*. This is indicated by the ability of the mutant to cause fully virulent infection in mice deficient in gp^{91phox} and iNOS, whilst WT mice inoculated with the *uvrB* mutant strain display attenuated infection and an intermediate phenotype is found when only iNOS is inactivated (Darwin and Nathan 2005). These observations confirm the importance of *uvrB* in defence against the products of phox *in vivo*.

In other bacterial species, it has been demonstrated that mice unable to generate ROIs display decreased survival compared with WT mice following infection with *Listeria monocytogenes* (Shiloh *et al.*, 1999). It has also been shown that ROIs are severely damaging to intracellular bacteria such as *Salmonella*

typhimurium. Despite the controversy surrounding ROIs as a host defence mechanism against Mtb, evidence does point towards a role, though potentially one that is neither fully understood nor defined.

It stands to reason that as ROI trigger such a plethora of destructive effects, their role can only be damaging to the pathogen. Interestingly, a new mechanism has been identified whereby it is pathogen derived NO which protects *Bacillus subtilis* from the effects of ROI (Gusarov and Nudler 2005). This research suggests that NO can suppress the reduction of free cysteine, which fuels the Fenton reaction and additionally reactivates catalase, an anti-oxidant. This has interesting connotations as it may be an additional mechanism for withstanding the effects of ROI and thus preventing damage and therefore the need for repair.

The results obtained in this study hint at a potential role for Rv2191 in the repair of damage sustained by exposure to ROI. However, the fact that the role appears specific to a small number of DNA damaging agents, as was demonstrated for UvrB (Darwin and Nathan 2005) may suggest that additional repair mechanisms and pathways repair damage sustained or that there are mechanisms in place to either prevent the damage occurring or reverse it.

In *E.coli*, Cho, a UvrC homologue, produces incisions on the 3' side of the lesion whilst UvrC incises on the 5' side. As Rv2191 is homologous to Cho, one can assume that its role is similar. It is hypothesised that certain types of damage, specifically bulky adducts, are more suitable for repair by Cho than by UvrC, although UvrC is thought to repair the majority of the damage (Moolenaar *et al.*,

2002). Prior to incision, the formation of the UvrB- DNA damage verification complex is essential for NER to function (Theis *et al.*, 2000). However, unlike UvrC, Cho does not require the UvrC binding domain of UvrB (Moolenaar *et al.*, 2002).

It is likely that as UvrC can incise much damage itself, this may account for the decrease in viability of the Rv2191 mutant following exposure to only certain types of damage, such as TBHP in the viability assay and menadione in the disc diffusion susceptibility assay. Presumably the damage incurred by exposure to the other damaging agents can be repaired by UvrC. This could be further investigated by constructing a UvrC/ Rv2191 double mutant and comparing viability with wild type and single mutant strains following exposure to various damaging agents.

Despite the fact that NER is strongly implicated in the repair of oxidative damage, there are many other pathways, albeit with their own limitations, that may also be able to repair this damage (Gros *et al.*, 2002). If the damage cannot be repaired by NER, it is probable that an alternative pathway such as BER could repair the damage.

6.7.2 Exposure to nitrosative damage *in vitro*

The effects of nitrosative damage were investigated by incubating the bacteria with acidified sodium nitrite, a generator of nitric oxide *in vitro*. The Rv2191 mutant displayed similar viability to the wild type strain following exposure to acidified sodium nitrite. This indicates that for the repair of the type of damage

induced by exposure to RNI at this concentration (3mM), Rv2191 does not play a role. As UvrB is involved in the repair of this type of damage (Darwin and Nathan 2005), implicating the NER pathway, this result points to the fact that Rv2191 is not an essential component in the repair of damage sustained by exposure to RNI. This result is supported by the fact that Cho (in *E. coli*) is thought to be an extra component of NER and repairs damage in addition to UvrC, which is the main incision protein for the pathway. To fully investigate this, again, a UvrC/Rv2191 mutant would have to be constructed and the experiment repeated. It is also possible that UvrB is able to work additionally as a component in other repair pathways or that the role of the incision enzymes (UvrC and Rv2191) can be carried out by different proteins.

However, the damage sustained by exposure to NO includes cross linked adducts, formed by one of the products of deaminated guanine, oxanine, reacting with polyamines and DNA binding proteins. These cross linked adducts are repaired potentially by the UvrABC complex and hence NER in *E. coli* (Nakano *et al.*, 2005). As this damage would be expected to be bulky, it could be hypothesized that Rv2191 would play a role here and this is supported by the result from the disc diffusion assay where the Rv2191 mutant displayed increased sensitivity to ofloxacin, which causes damage in the form of bulky adducts. It has also been demonstrated that in *E. coli*, the incision of the bulky adducts is dependent on the SOS response (Nakano *et al.*, 2005); the gene encoding Cho is upregulated as part of this response but *uvrC* is not. This is interesting as it further hints at the role of Rv2191 in Mtb survival during infection. In a previous study investigating the roles of *uvrA* and *uvrD* in survival, it was found that neither of these mutants

showed an increase in susceptibility to ofloxacin (Rand 2003), which suggests that they are not involved in the removal of bulky adducts. This could be further investigated by comparing the phenotype obtained in this study to that for a UvrC mutant and a UvrC/ Rv2191 mutant under the same conditions to further investigate the excision activity of NER during the repair of this kind of damage. It is possible that this investigation was not rigorous enough to demonstrate a potential phenotype for a gene that may only be involved in repair under certain specific conditions. It is also possible that the phenotype may be specific to other types of RNI. This could be investigated by exposing the mutant to a range of other RNI inducing compounds, such as GSNO and GSH. However, the lack of a role of Rv2191 in survival against nitrosative damage is supported by a study where it was not one of a number of genes shown to be hypersusceptible to acidified nitrite (Darwin *et al.*, 2003).

6.7.3 Exposure to Gamma radiation

There was no phenotype observed for the Rv2191 mutant upon exposure to ionizing radiation during the exponential phase of growth. This may support the hypothesis that the other repair pathways play a greater role in this kind of repair. However, it is also possible that if the damage incurred is relatively clean breaks and not damage with bulky adducts, this would potentially be preferentially repaired by UvrC and the other components of the NER pathway. In order to further investigate this and confirm a role for the NER pathway in this kind of damage, a UvrC/Rv2191 mutant could be constructed and subjected to ionizing radiation. Alternatively, to investigate the role of the entire pathway, an essential

component such as *uvrB* could be mutated and survival after exposure to ionizing radiation could be assessed.

NER plays an important role in the repair of UV damage to DNA. In *E. coli*, although Cho contributes to UV survival, it can not fully compensate in a UvrC mutant (Moolenaar *et al.*, 2002). This suggests a complementary role for Rv2191 in survival against damage associated with exposure to UV radiation. Therefore, an interesting further experiment could be to expose the Rv2191 mutant strain to UV light and investigate viability following this. An *Mtb uvrB* mutant was shown to be very sensitive to the effects of UV light (Darwin and Nathan 2005), confirming the role of the pathway in this kind of repair in *Mtb*, and it would be interesting to look at the role of Rv2191 here. If the mutant strain was more sensitive to UV light than the wild type, this would help to confirm the role of Rv2191 in NER. Constructing a double UvrC/ Rv2191 mutant and investigating the effect of UV light on viability compared to single mutants and wild type would also give a clearer picture of the exact roles of Rv2191 and UvrC in NER, and the potential overlap in these. *Mtb* must be able to withstand and repair damage incurred following exposure to UV radiation as it is exposed to it briefly during transmission from person to person.

6.7.4 Growth *in vivo*

Despite the phenotype seen for the Rv2191 mutant *in vitro*, there was no difference in growth between mutant and wild type strains *in vivo*, in either activated macrophages or in a mouse model of infection. Phenotypes *in vitro* are not always indicative of a change in growth pattern *in vivo* due to other host

factors involved in infection *in vivo*. Additionally, in human Mtb infection, only a small minority of the antimicrobial products produced by phagocytes which are delivered to the phagosome during infection *in vivo* are actually found within macrophages (Nathan and Shiloh 2000). Many of the antimicrobial proteins present in monocytes are lost during differentiation into phagocytes. Although ROI and RNI are of great importance in host defence, the role of these other proteins must not be discounted and for this reason, infection in activated macrophages may not give an entirely accurate picture of infection *in vivo*. Furthermore, the macrophages used in this study are derived from Balb/c mice and as mentioned in chapter 5, the immune response in mice is different to that in humans.

During the first 24h of infection, bacterial growth in macrophages is inhibited by superoxide and ROI are implicated in protection against infection with Mtb during the initial stages. Once the acquired immune response is generated cytokines are released and the bacterial load is reduced (Cooper *et al.*, 2000). It has been reported that Rv2191 showed a small induction in activated macrophages (Schnappinger *et al.*, 2003), which supports a potential role *in vivo*. Additionally, as it has been shown that UvrB is important in defence against the products of phox (Darwin and Nathan 2005); it would be interesting to look at survival of the Rv2191 mutant in Δ phox mice or phox-/iNOS- mice to compare the viability *in vivo* with a WT strain. This would give a better understanding of the potential role of the gene and pathway in defence against oxidative damage that might otherwise be masked by other repair pathways and/or defence mechanisms.

Survival of a UvrC/Rv2191 double mutant in mice and macrophages would enable investigation into the roles of these genes together and comparing this to the survival of the single mutants may clarify their individual roles in the pathway *in vivo*. This would help to further characterise the role of Rv2191 and its potential repair capacity *in vivo* in an infection model. However, as a *uvrB* mutant displayed only a slight attenuation in growth in WT mice (Darwin and Nathan 2005), which may suggest that the damage sustained during infection in mice is repaired by alternative DNA repair pathways.

6.7.5 Complementation

Complementation is a reliable method for restoring the phenotype of a mutant but there are problems associated with it. It is possible for the complementing construct to randomly integrate into a different site in the chromosome although this is very unlikely. The fact that the complemented strain restored the mutant phenotype back to wild type confirmed that the process had been successful. In the event that the complemented strain did not revert the mutant phenotype back to wild type, a simple test could have been performed. This involves a PCR reaction on the complement strain DNA to ensure that the gene is correctly restored. Additionally, complement RNA can be tested to ensure that the gene is expressed.

6.8 Concluding Remarks

The Rv2191 mutant displayed a decrease in viability following exposure to TBHP and increased susceptibility to menadione in a previous experiment. These compounds are both inducers of ROI. Despite this phenotype *in vitro*, no phenotype was demonstrated in an animal model.

Nonetheless, the results hint at a potential role for the gene in the repair of damage sustained as a result of exposure to ROI and support findings that the NER pathway is involved in the repair of damage sustained in this way (Darwin and Nathan 2005). It is possible that using the mouse model masked a phenotype that may have been seen in humans and it is also possible that a potential phenotype for the Rv2191 mutant may be more easily observed in Δ iNOS mice or macrophages, where the effects of phox are less masked.

It would be interesting to construct a UvrC/Rv2191 double mutant and compare the viability following oxidative damage to the single mutant and wild type strains. Given the role of NER in the repair of damage sustained by exposure to UV light and the fact that in *E. coli*, Cho is almost able to compensate for the loss of UvrC in this kind of repair (Moolenaar *et al.*, 2002), it would be worthwhile to carry out a UV susceptibility assay in Mtb, preferably with a double mutant strain. Additionally, microarray studies on the Rv2191 mutant could provide an insight into the regulation of other components of the NER pathway following exposure to DNA damage. In particular, this could be used to further investigate the effects of oxidative damage on a genomic level. It would also be interesting to investigate whether mutation of part of the NER pathway has an effect on other genes or

repair pathways and whether these are up or down regulated. This would be interesting as it would give a clearer picture of whether other genes may take over roles of DNA repair which may normally be carried out by Rv2191. A limitation of mutant studies is that the roles of other genes are unconfirmed and therefore the system can never be completely controlled.

In conclusion, the Rv2191 mutant is susceptible to specific types of DNA damage *in vitro*, but is not affected in its ability to grow *in vivo* in a mouse model. The data obtained in this study supports a role for Rv2191 in the repair of damage sustained as a result of exposure to oxidative damage.

7 General Discussion

The roles of selected genes involved in DNA repair were investigated using targeted mutation in order to gain an insight into their biological functions. This was then pursued via characterisation of the phenotypes of these mutant strains. Potential roles in DNA repair were investigated both *in vitro* using a variety of DNA damaging agents and *in vivo* using macrophages. For selected strains possible roles in pathogenesis were investigated using a mouse infection model.

7.1 Targeted mutation in *Mtb*

To investigate the importance of selected genes involved in DNA repair, mutants of the genes were constructed by exploiting the pathway of homologous recombination using a series of selection stages via antibiotic resistance cassettes and counterselectable markers. By mutating the gene, its role could be investigated in a number of ways. The effect of the absence of the gene on growth *in vitro*, as well as in macrophages, and for most strains in mice, was investigated. The role of the gene in DNA repair *in vitro* was investigated by comparing the effects of a variety of damaging conditions. These included exposure to filter discs impregnated with DNA damaging agents in a disc diffusion susceptibility assay, and exposure to ionizing radiation, where the *Mtb* gene knockouts were compared to the wild type parental strain. By collating all the different responses both *in vitro* and in a basic *in vivo* screen, it was hoped that a broad picture of the role of the gene in DNA repair and pathogenesis could be obtained. Following on from this, the roles of selected genes were investigated more thoroughly in a more sophisticated animal infection

model and with more probing *in vitro* experiments to further determine their function in DNA repair pathways.

DNA repair is an important element of bacterial survival *in vivo* and therefore may contribute to virulence. Recent studies have shown the importance of various DNA repair related Mtb genes in growth and persistence *in vivo* (Boshoff *et al.*, 2003; Darwin *et al.*, 2003; Sassetti and Rubin 2003), which supports the hypothesis that in order to create a successful infection, the bacteria must be able to repair damage incurred as a result of exposure to the host immune response. Following on from this, it appears that for bacteria such as Mtb, which spend extended periods of time in latency, alternative pathways of DNA repair may be important following the potential limitations of homologous recombination during this time of minimal DNA replication.

Mutants were constructed in Rv0937c and Rv0938, homologous to components of the Non-homologous End Joining pathway in eukaryotes; Rv2191, which shows homology to Cho in *E. coli*, a component of Nucleotide Excision Repair, and Rv3395c, a DNA damage inducible gene.

7.2 Rv3395c

Rv3395c has been shown to be DNA damage inducible and was the third most highly induced of all the DNA damage inducible genes identified by microarray (Rand *et al.*, 2003). It has also been demonstrated that Rv3395c is induced in activated macrophages but not naïve macrophages (Schnappinger *et al.*, 2003), supporting a potential role for the gene in DNA damage repair. Interestingly,

Rv3395c contains a RecA-like structural domain, which has been found in a large class of ATPases (Ye *et al.*, 2004). This domain appears to be present in other genes involved in DNA repair so it could be speculated that Rv3395c function is somehow related to the presence of this domain, which has been linked to mechanical work. Rv3395c is co-transcribed with Rv3394c (Rand *et al.*, 2003), which contains a *dinP* domain and belongs to the Y-family of error-prone DNA polymerases, implicated in DNA repair (Boshoff *et al.*, 2003). Additionally, *imuA* in *Caulobacter crescentus*, a homologue of Rv3395c, is involved in inducible mutagenesis (Galhardo *et al.*, 2005), and raises the question of a potential role for Rv3395c in damage induced mutation.

Growth of the Rv3395c mutant *in vitro* was unaffected and followed the same pattern as the wild type despite a small lag in growth between days 5 and 8. Growth in unactivated macrophages showed no significant differences between wild type and the mutant strain.

Results from the disc diffusion assay showed a statistically significant difference in growth compared to the wild type strain during exposure to mitomycin C. It has been demonstrated that Rv3395c is induced following exposure to mitomycin C (Davis *et al.*, 2002; Boshoff *et al.*, 2003; Rand *et al.*, 2003). Therefore, the results obtained here support that finding. Mitomycin C causes alkylation of DNA bases and crosslinking of complementary strands (Iyer and Szybalski 1963) so it can be speculated that either the protein encoded by Rv3395c plays a role in the repair of DNA cross links or perhaps the gene is involved in a regulatory mechanism which may cause upregulation of other genes involved in this kind of repair.

Growth of the Rv3395c mutant was no more affected than the wild type strain by exposure to bleomycin, menadione and ofloxacin, suggesting that the role of Rv3395c in DNA repair may be specific to certain types of damage. Bleomycin causes DNA double strand breaks and menadione causes damage similar to that incurred by exposure to oxidative stress so it could be postulated that Rv3395c is not directly involved in repair of these types of damage, although the range of compounds tested was presumably small compared with the range of damage incurred as a result of exposure to ROI *in vivo*. More probing experiments could investigate viability following exposure to DNA damage using a wider range of compounds generating oxidative stress. There remains the possibility of a role in survival following nitrosative stress, which could be investigated in the future, and additionally a role in growth *in vivo*, which is not always reflected by a phenotype *in vitro*. Following on from recent reports (Galhardo *et al.*, 2005), it would be interesting to investigate the role of Rv3395c in DNA damage induced mutation, which may be involved in the development of more resistant strains.

Results from this study suggest that the role of Rv3395c in DNA repair appears to be somewhat limited. Therefore, it was decided to exclude this mutant from further detailed investigation to concentrate on characterising the other mutant strains.

7.3 Rv2191

Rv2191 shows homology to Cho in *E. coli*, a recently discovered component of NER (Moolenaar *et al.*, 2002), encoded by an SOS inducible gene (Fernandez De Henestrosa *et al.*, 2000). As well as sharing an excinuclease domain with Cho,

Rv2191 has an additional exonuclease domain which implies a slight difference in function or an additional function specific to Mtb. This function is thought to involve degradation ability following the initial 3' incision, potentially in the absence or inactivation of UvrC. Here, the putative exonuclease activity of Cho would digest the area of the lesion in the 3' direction, leaving a 3' OH end, which could serve as a primer for repair synthesis (van Houten *et al.*, 2002). In NER, UvrC is recruited to the damage by UvrA and UvrB and incises the DNA on the 3' and 5' sides of the lesion. The 3' incision is made by Cho if access to the lesion by UvrC is blocked (Moolenaar *et al.*, 2002). The excision activity of both Cho and UvrC depends on the presence of UvrB to form a damage recognition complex with DNA (Theis *et al.*, 1999). As well as being present in *E. coli* and Mtb, Cho and UvrC coexist in bacterial species including *Listeria sp.* and *Clostridia sp.* (van Houten *et al.*, 2002).

The disc diffusion assays showed an increased inhibition of growth by ofloxacin and menadione for the Rv2191 mutant compared with the wild type. Ofloxacin affects DNA synthesis by inhibiting DNA gyrase, the enzyme which controls DNA supercoiling. Ofloxacin and gyrase subunitA covalently bind to the DNA ends, forming a complex (Higgins *et al.*, 1978) and inhibit religation of the broken strand, resulting in the formation of bulky adducts and a double strand break. It could be hypothesized that NER repairs this kind of damage with Rv2191 playing a role. In *E. coli*, Cho repairs damage on the 3' side of a lesion that is structurally obscured from UvrC (Moolenaar *et al.*, 2002). As exposure to ofloxacin results in the formation of bulky adducts, these could structurally obscure the repair by UvrC so the system would rely on Rv2191 to incise the damage on the 3' side.

When Rv2191 is inactivated, this is not possible, which could explain why the Rv2191 mutant is particularly susceptible to ofloxacin. The exonuclease domain present in Mtb Rv2191 could also play a role here and could be further investigated by complementing the mutant strain with Rv2191 lacking the exonuclease domain. Excision of bulky adducts was further investigated by examining the viability of the mutant strain following exposure to acidified sodium nitrite. However, Rv2191 did not show decreased viability under these conditions compared to the wild type strain. A recent study found that crosslinks in bacterial DNA incurred as a result of exposure to nitric oxide stress are repaired by the UvrABC complex (Nakano *et al.*, 2005). This is supported by recent work where it was demonstrated that a *uvrB* mutant was very sensitive to nitric oxide stress (Darwin and Nathan 2005). Despite the nucleotide excision repair pathway being implicated in this form of repair, it is possible that Rv2191 itself has a less important role, supported by the fact that UvrC may be able to excise most forms of damage.

Menadione, a redox recycling agent, causes the formation of oxygen free radicals and damages the bacterial DNA in a way similar to the damage incurred as a result of exposure to the oxidative stress response in activated macrophages. Bacterial DNA is damaged by reactive oxygen species, which cause the formation of single and double strand breaks and oxidised bases, a major consequence of exposure to oxidative stress. It is possible that Rv2191 plays a role in the repair of this kind of damage and following on from the initial observations, it was decided to investigate the viability of the Rv2191 mutant following exposure to a variety of compounds releasing ROIs. It was found that despite a clear decrease in

viability following exposure to a number of these DNA damaging agents compared with the wild-type and complemented strains, only the difference in viability following exposure to t-butyl hydroperoxide was statistically significant. Nonetheless, this confirms a role for Rv2191 in the repair of damage sustained as a result of exposure to ROIs, and supports findings by Darwin and Nathan (2005), where the NER pathway was demonstrated to be involved in repair of oxidative damage *in vivo*.

The Rv2191 mutant was no more susceptible than the wild type strain to gamma radiation. Gamma radiation induces double strand breaks in the DNA, as does bleomycin, so it could be surmised that Rv2191 does not play a role in the repair of DSBs directly. However, the NER pathway repairs damaged DNA strands primarily using UvrC for incision, so if Rv2191 is missing or inactivated it is likely that UvrC can repair the damage in most situations unless the damage was structurally obscured. This could be investigated by comparing the viability of the Rv2191 single mutant with the viability of a UvrC single mutant and a UvrC/Rv2191 double mutant after exposure to DNA damaging conditions. If both UvrC and Rv2191 depend on UvrA and UvrB, a *uvrA* or *uvrB* mutant would be very informative and such mutants already exist in Mtb.

Growth of the Rv2191 mutant *in vitro* showed no significant difference to that of the wild type. The same was observed for growth *in vivo*, both in activated macrophages and in a mouse infection model. Despite this, it has been reported that the expression of Rv2191 showed a small increase in activated macrophages (Schnappinger *et al.*, 2003), which suggests a potential role in survival *in vivo*.

although this was not observed in the infection models used in this study. Again, it is possible that the role of Rv2191 can be taken over by alternative genes or pathways in its absence. UvrC may be the obvious answer here. Construction of a UvrC/Rv2191 double mutant, and comparison of viability following DNA damage and during infection between the double and single mutant strains would be an interesting area to pursue in future.

7.4 Rv0937c and Rv0938

Rv0937c and Rv0938, homologous to the Ku and ATP-dependent ligase components of the non-homologous end joining pathway, were chosen for investigation in whole bacteria due to their reported role in this newly discovered repair pathway in Mtb from biochemical studies (Weller *et al.*, 2002). Mtb Ligase possesses a range of functions and the simpler NHEJ system in prokaryotes is just as capable of repairing double strand breaks that need processing as the more complex repair mechanism in eukaryotes (Weller *et al.*, 2004). As NHEJ plays an important role in the repair of DNA double strand breaks in eukaryotes, it would seem likely that the pathway is used for repairing similar damage in bacteria. This may suggest a role for the NHEJ pathway in pathogenesis as Mtb virulence is linked to its ability to withstand the human immune response, which damages bacterial DNA.

During the early stages of Mtb infection, the bacteria are engulfed by macrophages where they are able to survive and grow. Activated macrophages are stimulated by interferon gamma, produced by T cells, to produce reactive oxygen and nitrogen intermediates which damage the bacterial DNA in a number of ways,

including DNA breaks. The products of ROIs and RNIs must either be detoxified or the damage incurred must be repaired by one of many DNA repair pathways or proteins involved in repairing the damage directly. Therefore, it seems likely that a repair pathway which can repair double strand breaks will be implicated in the repair of DNA damage during infection and, hence, play a role in virulence. It has also been speculated that many of the bacteria with a functional NHEJ system either sporulate or spend periods of time in a dormant phase, thus raising the possibility that NHEJ may play a role in the repair of damage incurred during inactive phases (Weller *et al.*, 2002). Homologues of Ku are found in many bacteria including *B. subtilis*, *S. cereus*, *B. pertussis* (Aravind and Koonin 2001) and *S. aureus* (Wilson *et al.*, 2003). The presence of NHEJ repair has also been identified in biofilm forming bacteria such as *Pseudomonas sp.* (Wilson *et al.*, 2003), further implicating the role of the pathway in survival under a range of conditions. The fact that *Mtb* spend extended periods of time in latency, whilst still exposed to low level assault from the host immune response, suggests the importance of a functioning repair pathway that is able to repair double strand breaks without the need for a homologous template. This hypothesis could only be conclusively tested in a sophisticated animal model which is able to form granulomas and thus demonstrate a latent infection similar to that seen in humans.

Single mutants were constructed in Rv0937c and Rv0938, and a double Rv0937c/Rv0938 gene knockout was also made to completely delete the NHEJ pathway in *Mtb*.

The NHEJ mutants all showed a significantly increased susceptibility to bleomycin compared with the wild type strain. This supports a role for the selected genes, and thus the NHEJ pathway, in Mtb in the repair of double strand breaks. The Rv0937c/Rv0938 double mutant also showed a statistically significant increase in susceptibility to menadione compared with the wild type. Menadione induces DNA damage by mimicking conditions of oxidative stress and, therefore, the damage it induces includes double strand breaks. The fact that only the double mutant is more susceptible to this damaging agent suggests that under these conditions the Ku or Ligase component of the NHEJ pathway is sufficient to effect repair, perhaps in combination with an alternative protein. The possibility of another protein providing a 'back-up' function is supported by recent work where it was suggested that in mycobacteria another ATP-dependent ligase known as LigC (Rv3731) may be able to fulfill some of the functions of LigD (Rv0938), if LigD is absent (Gong *et al.*, 2005).

Mitomycin C had a significantly greater effect on the growth of the Rv0937c/Rv0938 mutant compared to the wild type. Mitomycin C causes inter-strand cross-links and it is possible that when these cross-links are removed by the excision processes the DNA becomes prone to breakage. If this is the case, these breaks would normally be repaired by either homologous recombination or NHEJ. So, if the NHEJ pathway is removed, the ability to repair the damage caused by mitomycin C will be decreased and the effect may be particularly marked in latency, where the role of homologous recombination is presumably limited.

Viability following exposure to ionizing radiation was also investigated as gamma radiation is an inducer of DNA double strand breaks. Surprisingly, there was no statistically significant difference between the wild type and the NHEJ mutants following exposure to gamma radiation during exponential or stationary phase, even though a potential role in the repair of double strand breaks was implicated by the fact that there was a larger area of inhibition observed around the bleomycin disc for all the NHEJ mutants compared with the wild type. A recent study reported a decrease in viability of NHEJ mutants in *Msm* after exposure to gamma radiation in stationary phase but not exponential phase (Pitcher *et al.*, 2007). It is possible that the dose of gamma radiation used here did not cause sufficient DNA breakage to readily detect a role for NHEJ in *Mtb*, although the amount used should have had some visible effect on survival. Using higher doses of radiation was limited by the equipment used in this study and is further complicated by the fact that work on *Mtb* is heavily restricted due to its category 3 biohazard status.

Alternatively, it is possible that NHEJ is involved in this kind of repair much later in stationary phase or in different conditions such as restricted oxygen or nutrient starvation. As *Msm* is not a pathogen, its requirements of repair are different to those of *Mtb* as is its exposure to damage. In another study, *Msm* single NHEJ mutants were demonstrated to be sensitive to high doses of gamma radiation only in late stationary phase and only when grown in minimal media (Stephanou *et al.*, 2007). Recent work suggests that successful repair via NHEJ during stationary phase may be attributed in part to the Rv0938 Pol domain (Pitcher *et al.*, 2007). Most prokaryotes possessing this domain are able to survive in stationary phase

and it is hypothesised that NHEJ polymerases may incorporate RNA to repair double strand breaks, when intracellular availability of dNTPs is low (Pitcher *et al.*, 2007). Thus, it remains possible, and perhaps likely, that the Mtb NHEJ pathway is important during persistence or dormancy. This could be further investigated by examining viability following exposure to gamma radiation much later in stationary phase or using models thought to mimic dormancy such as microaerobic conditions.

The NHEJ pathway is able to perform a multitude of DNA repair activities, which include template dependent fill-in of 5' overhangs, addition of single nucleotides at blunt ends via non-templated addition, and resection of 3' overhangs (Della *et al.*, 2004; Gong *et al.*, 2005). Therefore, it was decided to investigate the ability of the Mtb NHEJ mutant strains to repair complementary 5', 3' and blunt ends by comparing the capacity of the mutants to re-circularise a linearised plasmid with that of the wild type. The Rv0937c mutant showed a significant impairment in the ability to repair blunt, 5' and 3' breaks. The Rv0938 mutant also showed a similar decrease in ability to re-circularise the cut plasmid. These results are supported in part by data presented in a recent paper where it was shown that a Ligase mutant in Msm showed less of a decrease in repair of 5' and blunt ends compared with a Ku mutant in Msm (Gong *et al.*, 2005). Here, the ability to repair 5' ends was relatively uniform between all 3 mutant strains, and the repair of blunt ends was most significantly affected in the Rv0937c mutant strain. This could be explained if Rv0937c (Ku) were able to recruit an alternative ligase in the absence of Rv0938, although the severe impairment in repair in the Rv0938 mutant suggests

that the ligase encoded by Rv0938 is the major provider of this function in the wild-type.

In contrast, the Rv0937c/Rv0938 double mutant appeared to be slightly less affected than the single mutants for the repair of 3' overhangs, despite exhibiting a similar degree of impairment in the ability to repair blunt and 5' complementary DNA ends. This was, however, not statistically significant. This may suggest that in the single mutants, for the repair of overhanging 3' DNA, the remaining component of the pathway may interfere with a different repair pathway, for example homologous recombination, impairing its ability to repair the damage, and thus causing a further decrease in the ability of the strain to re-circularise the plasmid. Alternatively, it is possible that the remaining component physically blocks access to the DNA ends, thus disrupting repair. This result is supported by recent work in *Msm* (Stephanou *et al.*, 2007).

These results confirm a role for the NHEJ mutants in the repair of double strand breaks as the mutants were less able to re-circularise the plasmid compared with the wild type, and highlight the importance of the pathway, particularly in the repair of 5' and blunt ends. Additionally, repair of complementary 5' overhang and blunt DSBs has been demonstrated to be highly mutagenic in *Msm* (Gong *et al.*, 2005). The NHEJ pathway may be of equally low fidelity in *Mtb*, and may potentially play a role in the generation of strains better able to survive the damage incurred as a result of exposure to the immune response. This could be explored in further experiments.

Growth *in vitro* was not affected in any of the NHEJ mutants and this is supported by the results of a recent paper where it was found that the equivalent mutants in *Msm* had no effect on growth (Gong *et al.*, 2005). Growth in unactivated macrophages was also unaffected by the absence of the mutated genes. Interestingly, growth of all the NHEJ mutants was attenuated in activated macrophages compared with the wild type strain. This is an exciting finding as it confirms a role for the pathway in the repair of damage sustained *in vivo*.

However, growth in macrophages is not a conclusive indication of infection in an animal model and therefore, it was decided to investigate growth in a mouse infection model. Growth of all of the NHEJ mutants in mouse spleens followed a similar pattern to that of the wild type with no significant difference observed. Despite this, a significant decrease in spleen weight was observed in the Rv0937c mutant. In mouse lungs, there was a slight decrease in bacterial load for the Rv0937c and Rv0938 mutant at the last time point assessed. The double mutant appeared to phenocopy the wild type. The fact that the single mutants appear to be more affected than the double mutant, again support the hypothesis that in the single mutants, the remaining component of the pathway disrupts repair by alternative repair pathways in certain circumstances.

This result is interesting as it may implicate the NHEJ pathway in survival at late stages of infection, and it would be interesting to investigate later time points following infection to see if this effect became more marked and if any effect might then become apparent for the other mutants as the infection progressed. In addition, in order to investigate this more thoroughly in the future, a more

sophisticated animal model could be used. It would be interesting to use a different measure of pathogenesis, for example using a 'time to death' experiment, to investigate the effects of the mutants compared with the wild-type in the mouse model over a longer period of time. This would be useful if the mutants under investigation are involved in persistence or survival late in infection, as is predicted. It has previously been shown that an Mtb mutant in *dnaE2* (Boshoff *et al.*, 2003) is attenuated only at late time points following infection, with a 'time to death' analysis providing the clearest indication of this. Such experiments were not possible as part of the study presented here as they were not permitted on the animal licence under which the work was carried out.

In conclusion, the results obtained during the course of this study confirm the role of Rv0937c and Rv0938 in DNA damage repair by the decreased ability of the mutants to re-circularise linearised plasmids, and an increased susceptibility to various DNA damaging agents. The genes appear to be particularly important in the repair of double strand breaks induced by bleomycin and by RNI and ROI produced by activated macrophages, which supports the theory that NHEJ is an alternative pathway for the repair of this kind of DNA damage in Mtb (Weller *et al.*, 2002). The fact that all 3 NHEJ mutant strains displayed decreased growth in activated macrophages strongly implicates the pathway in survival of Mtb *in vivo*. This is supported by the attenuation of the Rv0937c and Rv0938 mutants during the later stages of infection in mice.

Despite the fact that the NHEJ pathway is simpler in prokaryotes it still appears to have an important role in DNA repair and may be the main repair pathway during

latency. This has exciting clinical implications as the NHEJ pathway could be used as a drug target during this time, where most antibiotics are restricted in their actions due to the fact they act on dividing bacteria only.

7.5 Future Perspectives

This study has characterised the roles of selected genes in DNA damage repair pathways, and it is clear from the results that Rv2191, Rv0937c and Rv0938 are involved in the repair of DNA damage, with a speculative role for Rv3395c in repair following exposure to mutagenic DNA damage supported.

Results obtained here confirm a role for NHEJ in the repair of DSBs, both *in vitro* and *in vivo* and so further investigation could provide information on a potential role of these genes in pathogenesis as well as survival. The function of the mutated genes could be further characterised by investigating viability following exposure to additional DNA damaging conditions. *In vitro* experiments could be repeated late in stationary phase to mimic the life stage of bacteria during latency. Survival and the effects of DNA damage in conditions of reduced oxygen, using the Wayne model (Wayne and Hayes 1996), or under nutrient starvation conditions could also be looked into to mimic the different stages of infection *in vivo*. Survival of the mutants in the Cornell mouse model (McCune *et al.*, 1956; McCune and Tompsett 1956), which induces an artificial state of latency, could also be investigated, which may be of particular relevance to the NHEJ mutants and may provide further clues into their roles. Above all, in order to confirm the results of these experiments, it would be advisable to construct complementing

strains for the NHEJ mutants, and repeat experiments where a phenotype was observed using these strains alongside the mutants. This would confirm that any phenotype observed is due to the mutation introduced.

Further work could be carried out on the pathways themselves by constructing mutants deficient in more than one pathway, for example a RecA/Rv0937c/Rv0938 mutant which would disable both the homologous recombination and non-homologous end joining pathways. This would enable a greater insight into the importance of the repair of double strand breaks and thus increased understanding of the pathways that repair them.

Despite the fact that no phenotype was observed for the Rv2191 mutant in an animal model, this work demonstrates its role in the repair of DNA *in vitro*. However, it is probable that for most types of damage, UvrC can work independently of Rv2191 and, therefore, the role of Rv2191 in infection is potentially quite limited. In order to confirm the functional relationship between UvrC and Rv2191, a double mutant could be constructed and viability compared to the single mutants. Viability following nitrosative stress could also be more rigorously pursued, using different compounds to initiate RNIs, especially as the NER pathway is implicated in the repair of this type of damage (Darwin and Nathan 2005).

7.6 Conclusion

To summarise, the experiments presented in this thesis have focused on characterising previously uncharacterised Mtb genes and investigating their roles in DNA repair.

The results show a potentially important role for NHEJ in the repair of damaged DNA both *in vitro* and in infection models. Combined with data available from homologous genes in Msm and knowledge of the pathway, it is tempting to speculate that NHEJ may play an important role in repair during persistence and thus may serve as a potential drug target for latent infection.

The role of Rv2191 was thoroughly investigated using viability assays following damage *in vitro*, and examining growth in an animal infection model. Despite no apparent phenotype in macrophages or mice, there was a clear and complemented decrease in viability observed following exposure to TBHP, confirming a role in the repair of damage following exposure to oxidative stress. This serves to further implicate the NER pathway in the repair of damage sustained as a result of exposure to ROI, but the functional role of Rv2191 during growth *in vivo*, as investigated by infection in macrophages and mice, appears to be replaceable.

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Appendix I Growth Media

L-broth

For 1 litre: 10g Bacto Trytone
 5g yeast extract
 1g NaCl

Modified Dubos medium

For 1 litre 1g KH₂PO₄
 6.25g Na₂HPO₄·12H₂O
 1.25g Na₃-citrate
 0.6g MgSO₄·7H₂O
 2g Asparagine
 2g Casamino acids
 5ml 10% Tween 80
 20ml 10% glycerol

Adjust to pH 7.2

Autoclave and add 40 ml Dubos Medium Albumin (Beckton Dickenson)

Appendix II Primers and probes

Table A: Primers used for mutant construction

Gene	Sequence
Rv0937c 5'F	ATCGCGGTGCCGGTCTCTGTAGGA
Rv0937c 5'R	GCTCTAGACGCGCTCGAAGGCCAACTCAAAC
Rv0937c 3'F	TAGGATCCGGTCTTGTTGCGCAGCGTGAAATG
Rv0937c 3'R	CGTAAGTGCCGGAGTCCCAGATGA
Rv0938 5'F	CGGTGGGACGCGGCATCTGACAT
Rv0938 5'R	GCTCTAGACGCCGACAACGCCAACTGCTTTTC
Rv0938 3' F	TAGAATTGCTGCGGGCCGGACAAGAAACC
Rv0938 3'R	CATCACCAGCAACGCCAGCAAGTC
Rv2191 5'F	GTAGCGGCCGCGACCTTGTT
Rv2191 5'R	GCTCTAGAGATCTGGGGCGGAATGCTGTGTTG
Rv2191 3'F	GCGAATTGCGCCGCGCTGCCCCGAGTTGAT
Rv2191 3'R	TCCGTTGCCCCGCCGATAC
Rv3395c 5'F	CGAGGTTTCAGCCGGTATGGATG
Rv3395c 5'R	GCTCTAGATGACCGCGCACCCGACAGCACTC
Rv3395c 3'F	GCGAATTGCCCCGTGCCCCGTCAAAAAG
Rv3395c 3'R	CGTTCGGCGCAAGTCATTCGTA

Table B: Probes used in Southern blots for confirming double crossovers

Gene	Sequence
Rv0937c F	TCCCATTATCGCCACCACCACTCA
Rv0937c R	GCACGCCGCCACACAGG
Rv0938 F	CGCGCTGTACCGGTTTTCCACT
Rv0938 R	GCCTCAGCCGCTTCCCGATACGAC
Rv2191 F	GCCAGGACGCCGGAGGAGACG
Rv2191 R	GCGCCGGTGGTAATCGACTGTGAC
Rv3395c F	CGACGCGGCCGAGACCTTCCT
Rv3395c R	CGTGCCGCAGCGTATCCAACC

Table C: Primers used for Rv2191 complement construction

Gene	Sequence
Rv2191 comp F	GATTTTCTAGAGCCAGGACGCCGGAGGAGAC
Rv2191 comp R	GAGGGTCTAGAGCGGCGGGCAGCGTGGATAAGTT